PROCESS AND MATERIALS FOR PRODUCTION OF GLUCOSAMINE

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part application of PCT Application No. PCT/US98/00800, filed January 14, 1998, which designates the United States. PCT/US98/00800 claims priority under 35 U.S.C. § 119(e) from U.S. Provisional Application Serial No. 60/035,494, filed January 14, 1997. Both PCT Application No. PCT/US98/00800 and U.S. Provisional Application Serial No. 60/035,494 are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to a method for producing glucosamine by fermentation. The present invention also relates to genetically modified strains of microorganisms useful for producing glucosamine.

BACKGROUND OF THE INVENTION

Amino sugars are usually found as monomer residues in complex oligosaccharides and polysaccharides. Glucosamine is an amino derivative of the simple sugar, glucose. Glucosamine and other amino sugars are important constituents of many natural polysaccharides. For example, polysaccharides containing amino sugars can form structural materials for cells, analogous to structural proteins.

Glucosamine is manufactured as a nutraceutical product with applications in the treatment of osteoarthritic conditions in animals and humans. The market for glucosamine is experiencing tremendous growth. Furthermore, significant erosion of the world market price for glucosamine is not expected.

Glucosamine is currently obtained by acid hydrolysis of chitin, a complex carbohydrate derived from *N*-acetyl-D-glucosamine. Alternatively, glucosamine can also be produced

by acid hydrolysis of variously acetylated chitosans. These processes suffer from poor product yields (in the range of 50% conversion of substrate to glucosamine). Moreover, the availability of raw material (i.e., a source of chitin, such as crab shells) is becoming increasingly limited. Therefore, there is a need in the industry for a cost-effective method for producing high yields of glucosamine for commercial sale and use.

SUMMARY OF THE INVENTION

One embodiment of the present invention relates to a method to produce glucosamine by fermentation of a microorganism. This method includes the steps of: (a) culturing in a fermentation medium a microorganism having a genetic modification in an amino sugar metabolic pathway; and (b) recovering a product produced from the step of culturing which is selected from the group of glucosamine-6-phosphate and glucosamine. Such an amino sugar metabolic pathway is selected from the group of a pathway for converting glucosamine-6-phosphate into another compound, a pathway for synthesizing glucosamine-6-phosphate, a pathway for transport of glucosamine or glucosamine-6-phosphate out of the microorganism, a pathway for transport of glucosamine into the microorganism, and a pathway which competes for substrates involved in the production of glucosamine-6-phosphate. The fermentation medium includes assimilable sources of carbon, nitrogen and phosphate. In a preferred embodiment, the microorganism is a bacterium or a yeast, and more preferably, a bacterium of the genus *Escherichia*, and even more preferably, *Escherichia coli*.

In one embodiment, the product can be recovered by recovering intracellular glucosamine-6-phosphate from the microorganism and/or recovering extracellular glucosamine from

the fermentation medium. In further embodiments, the step of recovering can include purifying glucosamine from the fermentation medium, isolating glucosamine-6-phosphate from the microorganism, and/or dephosphorylating the glucosamine-6-phosphate to produce glucosamine. In one embodiment, at least about 1 g/L of product is recovered.

In yet another embodiment, the step of culturing includes the step of maintaining the carbon source at a concentration of from about 0.5% to about 5% in the fermentation medium. In another embodiment, the step of culturing is performed at a temperature of from about 28°C to about 37°C. In yet another embodiment, the step of culturing is performed in a fermentor.

In one embodiment of the present invention, the microorganism has a modification in a gene which encodes a protein including, but not limited to, *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, *N*-acetyl-glucosamine-specific enzyme II^{Nag}, glucosamine-6-phosphate synthase, phosphoglucosamine mutase, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase, phosphofructokinase, enzyme II^{Glc} of the PEP:glucose PTS, EIIM,P/III^{Man} of the PEP:mannose PTS, and/or a phosphatase.

In another embodiment, the genetic modification includes a genetic modification which increases the action of glucosamine-6-phosphate synthase in the microorganism. Such a genetic modification includes the transformation of the microorganism with a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase to increase the action of glucosamine-6-phosphate synthase and/or to overexpress the glucosamine-6-phosphate synthase by the microorganism. In one embodiment, the recombinant nucleic acid molecule is operatively linked to a transcription control sequence. In a further embodiment, the recombinant nucleic

acid molecule is integrated into the genome of the microorganism. In yet another embodiment, the recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase has a genetic modification which increases the action of the synthase. Such genetic modifications can result in reduced glucosamine-6-phosphate product inhibition of the glucosamine-6-phosphate synthase, for example.

In one embodiment, a recombinant nucleic acid molecule of the present invention which comprises a nucleic acid sequence encoding a glucosamine-6-phosphate synthase encodes an amino acid sequence SEQ ID NO:16. In another embodiment, such a recombinant nucleic acid molecule comprises a nucleic acid sequence selected from the group of SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:15. Preferred recombinant nucleic acid molecules of the present invention include pKLN23-28, nglmS-28_{2184} and nglmS-28_{1830}.

Also included in the present invention are recombinant nucleic acid molecules encoding a glucosamine-6-phosphate synthase which comprises a genetic modification which increases the action of the glucosamine-6-phosphate synthase (i.e., a glucosamine-6-phosphate synthase homologue). Such a genetic modification can reduce glucosamine-6-phosphate product inhibition of the synthase, for example. In one embodiment, such a genetic modification in a recombinant nucleic acid molecule of the present invention which encodes a glucosamine-6-phosphate synthase results in at least one amino acid modification selected from the group of an addition, substitution, deletion, and/or derivatization of an amino acid residue of the glucosamine-6-phosphate synthase. In one embodiment, such an amino acid modification is in an amino acid sequence position in the modified protein (i.e., homologue) which corresponds to one or more of the following amino acid positions in amino acid sequence SEQ ID NO:16:

Ile(4), Ile(272), Ser(450), Ala(39), Arg(250), Gly(472), Leu(469). In another embodiment, such an amino acid modification is selected from the group of a substitution of: (a) an amino acid residue having an aliphatic hydroxyl side group for Ile(4); (b) an amino acid residue having an aliphatic hydroxyl side group for Ile(272); (c) an amino acid residue having an aliphatic side group for Ser(450); (d) an amino acid residue having an aliphatic hydroxyl side group for Ala(39); (e) an amino acid residue having a sulfur-containing side group for Arg(250); (f) an amino acid residue having an aliphatic hydroxyl side group for Gly(472); (g) an amino acid residue having an aliphatic side group for Leu(469); and, (h) combinations of (a)-(g).

In yet another embodiment of the present invention, an amino acid modification as described above is preferably a substitution selected from the group of: Ile(4) to Thr, Ile(272) to Thr, Ser(450) to Pro, Ala(39) to Thr, Arg(250) to Cys, Gly(472) to Ser, Leu(469) to Pro, and combinations thereof.

In another embodiment, a genetically modified recombinant nucleic acid molecule of the present invention comprises a nucleic acid sequence encoding glucosamine-6-phosphate synthase comprising an amino acid sequence selected from the group of SEQ ID NO:19, SEQ ID NO:22, SEQ ID NO:25, SEQ ID NO:28 or SEQ ID NO:31. In another embodiment, a genetically modified recombinant nucleic acid molecule of the present invention comprises a nucleic acid sequence selected from the group of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:29 and SEQ ID NO:30. Preferred genetically modified recombinant nucleic acid molecule of the present invention include pKLN23-49, pKLN23-54, pKLN23-124, pKLN23-149, pKLN23-

151, nglmS-49_{2184}, nglmS-49_{1830}, nglmS-54_{2184}, nglmS-54_{1830}, nglmS-124_{2184}, nglmS-124_{1830}, nglmS-149_{2184}, nglmS-149_{1830}, nglmS-151_{2184} and nglmS-151_{1830}.

Another embodiment of the present invention relates to a glucosamine-6-phosphate synthase which has glucosamine-6-phosphate synthase action, such synthase being encoded by a nucleic acid sequence having a genetic modification that results in increased glucosamine-6-phosphate synthase action. Such a nucleic acid sequence has been describe above with regard to recombinant nucleic acid molecules of the present invention.

Yet another embodiment of the present invention relates to a method to produce glucosamine by fermentation, such method comprising: (a) culturing in a fermentation medium comprising assimilable sources of carbon, nitrogen and phosphate, a genetically modified microorganism having increased glucosamine-6-phosphate synthase action, wherein the genetically modified microorganism is produced by a process comprising the steps of: 1) generating modifications in an isolated nucleic acid molecule comprising a nucleic acid sequence encoding glucosamine-6-phosphate synthase to create a plurality of modified nucleic acid sequences; (2) transforming microorganisms with the modified nucleic acid sequences to produce genetically modified microorganisms; (3)screening the genetically modified microorganisms for glucosamine-6-phosphate synthase action; and, (4) selecting the genetically modified microorganisms which have increased glucosamine-6-phosphate synthase action; ; and, (b) recovering the product. The step of culturing produces a product selected from the group of glucosamine-6-phosphate and glucosamine from the microorganism.

In another embodiment, a microorganism of the present invention has an additional genetic modification in genes encoding *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, *N*-acetyl-glucosamine-specific enzyme II^{Nag}, phosphoglucosamine mutase, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase, phosphofructokinase, Enzyme II^{Glc} of the PEP:glucose PTS, EIIM,P/III^{Man} of the PEP:mannose PTS, wherein the modification decreases the action of such proteins. In another embodiment, a microorganism of the present invention has an additional genetic modification in a gene encoding a phosphatase, wherein the modification increases the action of the phosphatase. In a preferred embodiment, a microorganism of the present invention has an additional genetic modification in the genes encoding the following proteins: *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and *N*-acetyl-glucosamine-specific enzyme II^{Nag}, such modifications including, but not limited to, a deletion of at least a portion of such genes.

Another embodiment of the present invention relates to a method to produce glucosamine by fermentation which includes the steps of (a) culturing an *Escherichia coli* transformed with a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase in a fermentation medium comprising assimilable sources of carbon, nitrogen and phcsphate to produce a product, and (b) recovering the product. The product includes intracellular glucosamine-6-phosphate which is recovered from the *Escherichia coli* and/or extracellular glucosamine which is recovered from the fermentation medium. In this embodiment, the recombinant nucleic acid molecule increases expression of the glucosamine-6-phosphate synthase by the *Escherichia coli*, and is operatively linked to a transcription control sequence. In one embodiment, the

recombinant nucleic acid molecule comprises a genetic modification which reduces glucosamine-6-phosphate product inhibition of the glucosamine-6-phosphate synthase. In another embodiment, the *Escherichia coli* has an additional genetic modification in at least one gene selected from the group of *nagA*, *nagB*, *nagC*, *nagD*, *nagE*, *manXYZ*, *glmM*, *pfkB*, *pfkA*, *glmU*, *glmS*, *ptsG* and/or a phosphatase gene. In yet another embodiment, the additional modification comprises a deletion of *nagA*, *nagB*, *nagC*, *nagD*, *nagE*, and a mutation in *manXYZ*, wherein the modification results in decreased enzymatic activity of *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and *N*-acetyl-glucosamine-specific enzyme II^{Nag}.

Yet another embodiment of the present invention relates to a microorganism for producing glucosamine by a biosynthetic process. The microorganism is transformed with a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase, wherein the recombinant nucleic acid molecule is operatively linked to a transcription control sequence. The recombinant nucleic acid molecule further comprises a genetic modification which increases the action of the glucosamine-6-phosphate synthase. The expression of the recombinant nucleic acid molecule increases production of the glucosamine by the microorganism. In a preferred embodiment, the recombinant nucleic acid molecule is integrated into the genome of the microorganism. In yet another embodiment, the microorganism has at least one additional genetic modification in a gene encoding a protein selected from the group consisting of *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, *N*-acetyl-glucosamine-specific enzyme II^{Nag}, phosphoglucosamine mutase, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase, phosphofructokinase, Enzyme II^{Glc} of the

PEP:glucose PTS, and/or EIIM,P/III^{Man} of the PEP:mannose PTS, wherein the genetic modification decreases the action of the protein. In another embodiment, the microorganism has a modification in a gene encoding a phosphatase, wherein the genetic modification increases the action of the phosphatase. In yet another embodiment, the microorganism has a modification in genes encoding *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and *N*-acetyl-glucosamine-specific enzyme II^{Nag}, wherein the genetic modification decreases enzymatic activity of the protein. In a preferred embodiment, the genetic modification is a deletion of at least a portion of the genes.

In a further embodiment, the microorganism is *Escherichia coli*, having a modification in a gene selected from the group of *nagA*, *nagB*, *nagC*, *nagD*, *nagE*, *manXYZ*, *glmM*, *pfkB*, *pfkA*, *glmU*, *ptsG* and/or a phosphatase gene. In one embodiment, such an *Escherichia coli* has a deletion of *nag* regulon genes, and in another embodiment, such an *Escherichia coli* has a deletion of *nag* regulon genes and a genetic modification in *manXYZ* genes such that the proteins encoded by the *manXYZ* genes have decreased action.

Yet another embodiment of the present invention is a microorganism as described above which produces at least about 1 g/L of glucosamine when cultured for about 10-60 hours at from about 28°C to about 37°C to a cell density of at least about 8 g/L by dry cell weight, in a pH 7.0 fermentation medium comprising: 14 g/L K_2HPO_4, 16 g/L KH_2PO_4, 1 g/L $Na_3Citrate{\cdot}2H_2O$, 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose, 10 mM $MgSO_4$, 1 mM $CaCl_2$, and from about 0.2mM to about 1 mM IPTG.

Another embodiment of the present invention is a microorganism for producing glucosamine by a biosynthetic process, which includes: (a) a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase operatively

linked to a transcription control sequence; and, (b) at least one genetic modification in a gene encoding a protein selected from the group of *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, *N*-acetyl-glucosamine-specific enzyme II^{Nag}, phosphoglucosamine mutase, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase, phosphofructokinase, Enzyme II^{Glc} of the PEP:glucose PTS, and/or EIIM,P/III^{Man} of the PEP:mannose PTS, wherein the genetic modification decreases the action of the protein. In another embodiment, the microorganism includes at least one genetic modification in a gene encoding a phosphatase, wherein the genetic modification increases the action of the phosphatase. Expression of the recombinant nucleic acid molecule increases the action of the glucosamine-6-phosphate synthase in the microorganism. In a further embodiment, the recombinant nucleic acid molecule is integrated into the genome of the microorganism.

BRIEF DESCRIPTION OF THE FIGURES OF THE INVENTION

Fig. 1 is a schematic representation of the pathways for the biosynthesis and catabolism of glucosamine and *N*-acetyl-glucosamine and their phosphorylated derivatives in *Escherichia coli*.

Fig. 2 is a schematic representation of the modifications to the pathways related to amino sugar metabolism for the overproduction of glucosamine in *Escherichia coli*.

Fig. 3 is a schematic representation of the production of *Escherichia coli* strains containing combinations of the *manXYZ*, *ptsG*, and *Δnag* mutations.

Fig. 4 is a line graph illustrating the effects on glucosamine accumulation of feeding additional glucose and ammonium sulfate to cultures.

Fig. 5 is a line graph which shows that glucosamine-6-phosphate synthase is inhibited by glucosamine-6-phosphate and glucosamine.

Fig. 6 is a line graph illustrating product inhibition of glucosamine-6-phosphate synthase activity in mutant *glmS* clones.

Fig. 7 is a schematic representation of the strategy for constructions of *Escherichia coli* strains containing mutant *glmS* genes.

Fig. 8 is a line graph illustrating product inhibition of glucosamine-6-phosphate synthase in *Escherichia coli* strains with integrated mutant *glmS* genes.

Fig. 9 is a line graph showing glucosamine production in mutant *Escherichia coli* strains with integrated mutant *glmS* genes.

Fig. 10 is a line graph showing inhibition of glucosamine-6-phosphate synthase in glucosamine-producing strains.

Fig. 11A is a line graph showing the thermal stability at 45°C of glucosamine-6-phosphate synthase in glucosamine-producing strains.

Fig. 11B is a line graph illustrating the thermal stability at 50°C of glucosamine-6-phosphate synthase in glucosamine-producing strains.

Fig. 12 is a line graph showing the effect of IPTG concentration on glucosamine production.

Fig. 13 is a line graph demonstrating the effects of IPTG concentration and temperature on glucosamine production.

Fig. 14A is a line graph illustrating growth and glucosamine production by glucosamine-producing strain 2123-54 at 30°C.

Fig. 14B is a line graph illustrating growth and glucosamine production by glucosamine-producing strain 2123-54 at 37°C.

Fig. 15A is a line graph showing glucosamine production by strain 2123-49 at 30°C.

Fig. 15B is a line graph showing glucosamine production by strain 2123-124 at 30°C.

Fig. 16A is a line graph illustrating glucosamine production by a glucosamine-producing strain in a glucose limited fermentor at 37°C.

Fig. 16B is a line graph illustrating glucosamine production by a glucosamine-producing strain in a glucose limited fermentor at 30°C.

Fig. 16A is a line graph illustrating glucosamine production by a glucosamine-producing strain in a glucose excess fermentor at 30°C.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a biosynthetic method for producing glucosamine. Such a method includes fermentation of a genetically modified microorganism to produce glucosamine. The present invention also relates to genetically modified microorganisms, such as strains of *Escherichia coli*, useful for producing glucosamine. As used herein, the terms glucosamine and *N*-glucosamine can be used interchangeably. Similarly, the terms glucosamine-6-phosphate and *N*-glucosamine-6-phosphate can be used interchangeably.

Glucosamine can also be abbreviated as GlcN and glucosamine-6-phosphate can also be abbreviated as GlcN-6-P.

The novel method of the present invention for production of glucosamine by fermentation is inexpensive and can produce a yield of glucosamine that exceeds the yield per cost of glucosamine produced by current hydrolysis methods. In addition, by using a genetically modified microorganism as described herein, the method of the present invention can be easily modified to adapt to particular problems or changing needs relative to the production of glucosamine.

The amino sugars, *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN), are fundamentally important molecules in microorganisms, because they are the precursors for the biosynthesis of major macromolecules, and in particular, glycoconjugates (i.e., macromolecules containing covalently bound oligosaccharide chains). For example, in *Escherichia coli*, *N*-acetylglucosamine and glucosamine are precursors for two macromolecules of the cell envelope, peptidoglycan and lipopolysaccharide. Mutations that block the biosynthesis of peptidoglycan or lipopolysaccharide are lethal, resulting in loss of integrity of the cell envelope and ultimately in cell lysis.

One embodiment of the present invention relates to a method to produce glucosamine by fermentation of a microorganism. This method includes the steps of (a) culturing in a fermentation medium a microorganism having a genetic modification in an amino sugar metabolic pathway which includes: a pathway for converting glucosamine-6-phosphate into another compound, a pathway for synthesizing glucosamine-6-phosphate, a pathway for transport of glucosamine or glucosamine-6-phosphate out of said microorganism, a pathway

for transport of glucosamine into said microorganism, and a pathway which competes for substrates involved in the production of glucosamine-6-phosphate, to produce a product which can include intracellular glucosamine-6-phosphate and/or extracellular glucosamine from the microorganism; and (b) recovering the product by recovering intracellular glucosamine-6-phosphate from the microorganism and/or recovering extracellular glucosamine from the fermentation medium. The fermentation medium includes assimilable sources of carbon, nitrogen and phosphate.

Another embodiment of the present invention relates to a method to produce glucosamine by fermentation. Such method includes the steps of: (a) culturing in a fermentation medium comprising assimilable sources of carbon, nitrogen and phosphate, an *Escherichia coli* transformed with a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase operatively linked to a transcription control sequence; and (b) recovering a product selected from the group of glucosamine-6-phosphate and glucosamine. The recombinant nucleic acid molecule increases expression of the glucosamine-6-phosphate synthase by the *Escherichia coli*. In a further embodiment, the recombinant nucleic acid molecule comprises a genetic modification which reduces glucosamine-6-phosphate product inhibition of the glucosamine-6-phosphate synthase. In yet another embodiment, the *Escherichia coli* has an additional genetic modification in at least one gene selected from the group of *nagA*, *nagB*, *nagC*, *nagD*, *nagE*, *manXYZ*, *glmM*, *pfkB*, *pfkA*, *glmU*, *glmS*, *ptsG* and/or a phosphatase gene.

To produce significantly high yields of glucosamine by the fermentation method of the present invention, a microorganism is genetically modified to enhance production of

glucosamine. As used herein, a genetically modified microorganism, such as *Escherichia coli*, has a genome which is modified (i.e., mutated or changed) from its normal (i.e., wild-type or naturally occurring) form. Genetic modification of a microorganism can be accomplished using classical strain development and/or molecular genetic techniques. Such techniques are generally disclosed, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press. The reference Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety. Additionally, techniques for genetic modification of a microorganism are described in detail in the Examples section. A genetically modified microorganism can include a natural genetic variant as well as a microorganism in which nucleic acid molecules have been inserted, deleted or modified (i.e., mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that such modifications provide the desired effect within the microorganism. According to the present invention, a genetically modified microorganism includes a microorganism that has been modified using recombinant technology. As used herein, genetic modifications which result in a decrease in gene expression, in the function of the gene, or in the function of the gene product (i.e., the protein encoded by the gene) can be referred to as inactivation (complete or partial), deletion, interruption, blockage or down-regulation of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such gene, can be the result of a complete deletion of the gene (i.e., the gene does not exist, and therefore the protein does not exist), a mutation in the gene which results

in incomplete or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no enzymatic activity or action). Genetic modifications which result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, or up-regulation of a gene.

In one embodiment of the present invention, a genetic modification of a microorganism increases or decreases the action of a protein involved in an amino sugar metabolic pathway according to the present invention. Such a genetic modification includes any type of modification and specifically includes modifications made by recombinant technology and by classical mutagenesis. For example, in one embodiment, a microorganism of the present invention has a genetic modification that increases the action of glucosamine-6-phosphate synthase. It should be noted that reference to increasing the action (or activity) of glucosamine-6-phosphate synthase and other enzymes discussed herein refers to any genetic modification in the microorganism in question which results in increased functionality of the enzymes and includes higher activity of the enzymes (e.g., specific activity or *in vivo* enzymatic activity), reduced inhibition or degradation of the enzymes and overexpression of the enzymes. For example, gene copy number can be increased, expression levels can be increased by use of a promoter that gives higher levels of expression than that of the native promoter, or a gene can be altered by genetic engineering or classical mutagenesis to increase the action of an enzyme. Examples of nucleic acid molecules encoding glucosamine-6-phosphate synthase which have been genetically modified to increase the action of the

glucosamine-6-phosphate synthase are described in the Examples section. Similarly, reference to decreasing the action of enzymes discussed herein refers to any genetic modification in the microorganism in question which results in decreased functionality of the enzymes and includes decreased activity of the enzymes (e.g., specific activity), increased inhibition or degradation of the enzymes and a reduction or elimination of expression of the enzymes. For example, the action of an enzyme of the present invention can be decreased by blocking or reducing the production of the enzyme, reducing enzyme activity, or inhibiting the activity of the enzyme. Blocking or reducing the production of an enzyme can include placing the gene encoding the enzyme under the control of a promoter that requires the presence of an inducing compound in the growth medium. By establishing conditions such that the inducer becomes depleted from the medium, the expression of the gene encoding the enzyme (and therefore, of enzyme synthesis) could be turned off. Blocking or reducing the activity of an enzyme could also include using an excision technology approach similar to that described in U.S. Patent No. 4,743,546, incorporated herein by reference. To use this approach, the gene encoding the enzyme of interest is cloned between specific genetic sequences that allow specific, controlled excision of the gene from the genome. Excision could be prompted by, for example, a shift in the cultivation temperature of the culture, as in U.S. Patent No. 4,743,546, or by some other physical or nutritional signal.

An amino sugar is an amino derivative of a saccharide (e.g., a saccharide having an amino group in place of a hydroxyl group). According to the present invention, an amino sugar metabolic pathway is any biochemical pathway involved in, or affecting, the biosynthesis, anabolism or catabolism of an amino sugar. As used herein, amino sugar metabolic

pathways include pathways involved in the transport of amino sugars and their precursors into and out of a cell, and can also include biochemical pathways which compete for substrates involved in the biosynthesis or catabolism of an amino sugar. For example, the immediate precursor to one of the earliest formed amino sugars is fructose-6-phosphate (F-6-P), which, in a biochemical reaction with glutamine (Gln, the amino group donor), forms glucosamine-6-phosphate. Fructose-6-phosphate is also an intermediate in the glycolysis pathway. Therefore, the glycolytic pathway competes with the glucosamine-6-phosphate biosynthetic pathway by competing for a substrate, fructose-6-phosphate. In addition, glucosamine-6-phosphate can be converted to other amino sugars and form constituents in various macromolecules by a series of biochemical reactions. As such, the fructose-6-phosphate/glucosamine-6-phosphate pathway, the fructose-6-phosphate glycolytic pathway, to the extent that it affects the biosynthesis of glucosamine-6-phosphate, and the glucosamine-6-phosphate/macromolecule biosynthesis pathway are all considered to be amino sugar metabolic pathways in the present invention.

In general, a microorganism having a genetically modified amino sugar metabolic pathway has at least one genetic modification, as discussed above, which results in a change in one or more amino sugar metabolic pathways as described above as compared to a wild-type microorganism cultured under the same conditions. Such a modification in an amino sugar metabolic pathway changes the ability of the microorganism to produce an amino sugar. According to the present invention, a genetically modified microorganism preferably has an enhanced ability to produce glucosamine compared to a wild-type microorganism cultured under the same conditions. An

amino sugar metabolic pathway which affects the production of glucosamine can generally be categorized into at least one of the following kinds of pathways: (a) pathways for converting glucosamine-6-phosphate into other compounds, (b) pathways for synthesizing glucosamine-6-phosphate, (c) pathways for transporting glucosamine into a cell, (d) pathways for transporting glucosamine or glucosamine-6-phosphate out of a cell, and (e) pathways which compete for substrates involved in the production of glucosamine-6-phosphate.

A genetically modified microorganism useful in a method of the present invention typically has at least one modified gene involved in at least one amino sugar metabolic pathway which results in (a) reduced ability to convert glucosamine-6-phosphate into other compounds (i.e., inhibition of glucosamine-6-phosphate catabolic or anabolic pathways), (b) an enhanced ability to produce (i.e., synthesize) glucosamine-6-phosphate, (c) a reduced ability to transport glucosamine into the cell, (d) an enhanced ability to transport glucosamine-6-phosphate or glucosamine out of the cell, and/or (e) a reduced ability to use substrates involved in the production of glucosamine-6-P for competing biochemical reactions.

It is to be understood that the present invention discloses a method comprising the use of a microorganism with an ability to produce commercially useful amounts of glucosamine in a fermentation process (i.e., preferably an enhanced ability to produce glucosamine compared to a wild-type microorganism cultured under the same conditions). This method is achieved by the genetic modification of one or more genes encoding a protein involved in an amino sugar metabolic pathway which results in the production (expression) of a

protein having an altered (e.g., increased or decreased) function as compared to the corresponding wild-type protein. Such an altered function enhances the ability of the genetically engineered microorganism to produce glucosamine. It will be appreciated by those of skill in the art that production of genetically modified microorganisms having a particular altered function as described elsewhere herein (e.g., an enhanced ability to produce glucosamine-6-phosphate) such as by the specific selection techniques described in the Examples, can produce many organisms meeting the given functional requirement, albeit by virtue of a variety of different genetic modifications. For example, different random nucleotide deletions and/or substitutions in a given nucleic acid sequence may all give rise to the same phenotypic result (e.g., decreased action of the protein encoded by the sequence). The present invention contemplates any such genetic modification which results in the production of a microorganism having the characteristics set forth herein.

For a variety of microorganisms, many of the amino sugar metabolic pathways have been elucidated. In particular, pathways for the biosynthesis and catabolism of glucosamine and *N*-acetylglucosamine and their phosphorylated derivatives have been elucidated in *Escherichia coli*. These pathways include the multiple transport systems for the utilization of these amino sugars as carbon sources. Genes encoding the enzymes and proteins directly related to the transport, catabolism and biosynthesis of amino sugars in *Escherichia coli* have been cloned and sequenced. In addition, mutant strains of *Escherichia coli* blocked in substantially every step of amino sugar metabolism have been isolated. The known

pathways for amino sugar metabolism for *Escherichia coli* are illustrated in Fig. 1.

As will be discussed in detail below, even though many of the pathways and genes involved in the amino sugar metabolic pathways have been elucidated, until the present invention, it was not known which of the many possible genetic modifications would be necessary to generate a microorganism that can produce commercially significant amounts of glucosamine. Indeed, the present inventors are the first to design and engineer a glucosamine-producing microorganism that has glucosamine production capabilities that far exceed the glucosamine production capability of any known wild-type or mutant microorganism. The present inventors are also the first to appreciate that such a genetically modified microorganism is useful in a method to produce glucosamine for commercial use.

A microorganism to be used in the fermentation method of the present invention is preferably a bacterium or a yeast. More preferably, such a microorganism is a bacterium of the genus *Escherichia*. *Escherichia coli* is the most preferred microorganism to use in the fermentation method of the present invention. Particularly preferred strains of *Escherichia coli* include K-12, B and W, and most preferably, K-12. Although *Escherichia coli* is most preferred, it is to be understood that any microorganism that produces glucosamine and can be genetically modified to enhance production of glucosamine can be used in the method of the present invention. A microorganism for use in the fermentation method of the present invention can also be referred to as a production organism.

The amino sugar metabolic pathways of the microorganism, *Escherichia coli,* will be addressed as specific embodiments of the present invention are described below. It will be appreciated that other microorganisms and in particular, other bacteria, have similar amino sugar metabolic pathways and genes and proteins having similar structure and function within such pathways. As such, the principles discussed below with regard to *Escherichia coli* are applicable to other microorganisms.

In one embodiment of the present invention, a genetically modified microorganism includes a microorganism which has an enhanced ability to synthesize glucosamine-6-phosphate. According to the present invention, "an enhanced ability to synthesize" a product refers to any enhancement, or up-regulation, in an amino sugar metabolic pathway related to the synthesis of the product such that the microorganism produces an increased amount of the product compared to the wild-type microorganism cultured under the same conditions. In one embodiment of the present invention, enhancement of the ability of a microorganism to synthesize glucosamine-6-phosphate is accomplished by amplification of the expression of the glucose-6-phosphate synthase gene, which in *Escherichia coli* is the *glmS* gene, the product of which is glucosamine-6-phosphate synthase. Glucosamine-6-phosphate synthase catalyzes the reaction in which fructose-6-phosphate and glutamine form glucosamine-6-phosphate and glutamic acid. Amplification of the expression of glucosamine-6-phosphate synthase can be accomplished in *Escherichia coli*, for example, by introduction of a recombinant nucleic acid molecule encoding the *glmS* gene.

Overexpression of *glmS* is crucial for the intracellular accumulation of glucosamine-6-phosphate and ultimately for production of glucosamine, since the level of glucosamine-6-phosphate synthase in the cell will control the redirection of carbon flow away from glycolysis and into glucosamine-6-phosphate synthesis. The *glmS* gene is located at 84 min on the *Escherichia coli* chromosome, and sequence analysis of this region of the chromosome reveals that *glmS* resides in an operon with the *glmU* gene, which encodes the bifunctional enzyme, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase. Glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase functions within the amino sugar metabolic pathway in which glucosamine-6-phosphate is incorporated, through a series of biochemical reactions, into macromolecules. No obvious promoter sequence is detected upstream of *glmS;* transcription of the *glmUS* operon is initiated from two promoter sequences upstream of *glmU.* Thus, it is preferred that the *glmS* gene be cloned under control of an artificial promoter. The promoter can be any suitable promoter that will provide a level of *glmS* expression required to maintain a sufficient level of glucosamine-6-phosphate synthase in the production organism. Preferred promoters are constitutive (rather than inducible) promoters, since the need for addition of expensive inducers is therefore obviated. Such promoters include normally inducible promoter systems that have been made functionally constitutive or "leaky" by genetic modification, such as by using a weaker, mutant repressor gene. Particularly preferred promoters to be used with *glmS* are *lac*, λP_L and T7. The gene dosage (copy number) of *glmS* can be varied according to the requirements for

maximum product formation. In one embodiment, the recombinant *glmS* gene is integrated into the *E. coli* chromosome.

Therefore, it is an embodiment of the present invention to provide a microorganism, such as *E. coli*, which is transformed with a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a glucosamine-6-phosphate synthase, which in *E. coli*, for example, is encoded by the *glmS* gene. Preferred recombinant nucleic acid molecules comprising such a nucleic acid sequence include recombinant nucleic acid molecules comprising a nucleic acid sequence which encodes a glucosamine-6-phosphate synthase comprising an amino acid sequence SEQ ID NO:16. Other preferred recombinant nucleic acid molecules of the present invention include nucleic acid molecules which comprise a nucleic acid sequence selected from the group of SEQ ID NO:13, SEQ ID NO:14 and/or SEQ ID NO:15. Particularly preferred recombinant nucleic acid molecules of the present invention include nucleic acid molecules comprising nucleic acid molecules nglmS-28_{2184} and/or nglmS-28_{1830}. One recombinant molecule of the present invention, referred to herein as plasmid pKLN23-28, includes SEQ ID NOs:13, 14 and 15 and is particularly useful for expressing glucosamine-6-phosphate synthase in a microorganism. The above identified nucleic acid molecules represent nucleic acid molecules comprising wild-type (i.e., naturally occurring or endogenous) nucleic acid sequences encoding glucosamine-6-phosphate synthase proteins. Genetically modified nucleic acid molecules which include nucleic acid sequences encoding homologues (i.e., modified and/or mutated) glucosamine-6-phosphate synthase proteins are also encompassed by the present invention and are described in detail below.

The reported K_m's of glucosamine-6-phosphate synthase from *Escherichia coli* are 2mM and 0.4mM for fructose-6-phosphate and glutamine, respectively. These are relatively high values (i.e., the affinity of the enzyme for its substrates is rather weak). It is therefore another embodiment of the present invention to provide a microorganism having a glucosamine-6-phosphate synthase with improved affinity for its substrates. A glucosamine-6-phosphate synthase with improved affinity for its substrates can be produced by any suitable method of genetic modification or protein engineering. For example, computer-based protein engineering can be used to design a glucosamine-6-phosphate synthase protein with greater stability and better affinity for its substrate. See for example, Maulik et al., 1997, *Molecular Biotechnology: Therapeutic Applications and Strategies*, Wiley-Liss, Inc., which is incorporated herein by reference in its entirety.

White (1968, *Biochem. J.*, 106:847-858) first demonstrated that glucosamine-6-phosphate synthase was inhibited by glucosamine-6-phosphate. The present inventors determined that this inhibition was a key factor which limits glucosamine accumulation in glucosamine production strains of the present invention, which have been designed for commercial use. Therefore, it is yet another embodiment of the present invention to provide a microorganism having a glucosamine-6-phosphate synthase with reduced glucosamine-6-phosphate product feedback inhibition. A glucosamine-6-phosphate synthase with reduced product inhibition can be a mutated (i.e., genetically modified) glucosamine-6-phosphate synthase gene, for example, and can be produced by any suitable method of genetic modification. For example, a recombinant nucleic

acid molecule encoding glucosamine-6-phosphate synthase can be modified by any method for inserting, deleting, and/or substituting nucleotides, such as by error-prone PCR. In this method, the gene is amplified under conditions that lead to a high frequency of misincorporation errors by the DNA polymerase used for the amplification. As a result, a high frequency of mutations are obtained in the PCR products. This method is described in detail in Example 5. The resulting glucosamine-6-phosphate synthase gene mutants can then be screened for reduced product inhibition by testing the mutant genes for the ability to confer increased glucosamine production onto a test microorganism, as compared to a microorganism carrying the non-mutated recombinant glucosamine-6-phosphate synthase nucleic acid molecule. It should be noted that decreased product inhibition of glucosamine-6-phosphate synthase typically results in a glucosamine-6-phosphate synthase with increased action, even when the specific activity of the enzyme is remains the same, or actually is decreased, relative to a naturally occurring glucosamine-6-phosphate enzyme. Therefore, it is an embodiment of the present invention to produce a genetically modified glucosamine-6-phosphate synthase with increased action and increased *in vivo* enzymatic activity, which has unmodified or even decreased specific activity as compared to a naturally occurring glucosamine-6-phosphate synthase. Also encompassed by the present invention are genetically modified glucosamine-6-phosphate synthases with increased specific activity.

Therefore, it is an embodiment of the present invention to provide a microorganism, such as *E. coli*, which is transformed with a genetically modified recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a

mutant, or homologue, glucosamine-6-phosphate synthase protein. Such glucosamine-6-phosphate synthase proteins can be referred to herein as glucosamine-6-phosphate synthase homologues. Protein homologues are described in detail below. Preferred recombinant nucleic acid molecules comprising such a nucleic acid sequence include recombinant nucleic acid molecules comprising a nucleic acid sequence which encodes a glucosamine-6-phosphate synthase comprising an amino acid sequence selected from the group of SEQ ID NO:19, SEQ ID NO:22, SEQ ID NO:25, SEQ ID NO:28 and/or SEQ ID NO:31. Other preferred recombinant nucleic acid molecules comprise a nucleic acid sequence selected from the group of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:29 and/or SEQ ID NO:30. Particularly preferred genetically modified recombinant nucleic acid molecules useful in the present invention include nucleic acid molecules comprising nucleic acid molecules selected from the group of nglmS-49_{2184}, nglmS-49_{1830}, nglmS-54_{2184}, nglmS-54_{1830}, nglmS-124_{2184}, nglmS-124_{1830}, nglmS-149_{2184}, nglmS-149_{1830}, nglmS-151_{2184} and nglmS-151_{1830}. Plasmids pKLN23-49, pKLN23-54, pKLN23-124, pKLN23-149 and pKLN23-151 are recombinant nucleic acid molecules of the present invention which are particularly useful for expressing glucosamine-6-phosphate synthase homologues in a microorganism.

An adequate intracellular supply of glutamine (Gln) is critical for the glucosamine-6-phosphate synthase reaction. Inspection of the synthetic and degradative pathways for glucosamine-6-phosphate reveals the presence of a potential futile cycle whereby continuous interconversion of fructose-6-phosphate and glucosamine-6-phosphate results in wasteful depletion of glutamine. In one embodiment of the present

invention, the supply of glutamine can be increased either by genetic modification of the production organism to increase glutamine production in the cell, or by modifying the fermentation medium (i.e., adding glutamine to the fermentation medium), to ensure that the supply of glutamine will not limit the production of glucosamine-6-phosphate.

In another embodiment of the present invention, the potential futile cycling of fructose-6-phosphate and glucosamine-6-phosphate is addressed by inhibiting, or blocking, the reverse reaction in which glucosamine-6-phosphate is converted into fructose-6-phosphate. In this embodiment, a microorganism is genetically modified to have an inactivation or deletion of the gene which catalyzes this conversion, glucosamine-6-phosphate deaminase, which in *Escherichia coli* is the *nagB* gene. *nagB* is one of several *nag* genes which are part of the *nag* regulon. The *nag* genes involved in the degradation of glucosamine and *N*-acetyl-glucosamine exist as a regulon located at 15 min on the *Escherichia coli* chromosome. In another embodiment, the entire *nag* regulon is inactivated or deleted. The advantages of deleting the entire *nag* regulon are discussed in detail below.

As discussed above, overproduction of glucosamine-6-phosphate synthase results in diversion of fructose-6-phosphate synthesis to glucosamine-6-phosphate synthesis. However, many other enzymes can compete for the substrate, fructose-6-phosphate. Therefore, one embodiment of the present invention includes a microorganism in which these competitive side reactions are blocked. In a preferred embodiment, a microorganism having complete or partial inactivation of the gene encoding phosphofructokinase is provided. The second step in the glycolytic pathway is the

conversion of fructose-6-phosphate to fructose-1,6-diphosphate by phosphofructokinase, which in *Escherichia coli* exists as two isozymes encoded by the *pfkA* and *pfkB* genes. Complete or partial inactivation of either the *pfkA* or *pfkB* genes slows the entry of fructose-6-phosphate into the glycolytic pathway and enhances the conversion of fructose-6-phosphate to glucosamine-6-phosphate. As used herein, inactivation of a gene can refer to any modification of a gene which results in a decrease in the activity (i.e., expression or function) of such a gene, including attenuation of activity or complete deletion of activity.

In a further embodiment of the present invention, a genetically modified microorganism has a decreased ability to convert glucosamine-6-phosphate into other compounds. Inactivation of glucosamine-6-phosphate deaminase, as described above, represents one such modification, however, glucosamine-6-phosphate serves as a substrate for other biochemical reactions. The first committed step in the pathway leading to production of macromolecules such as lipopolysaccharide and peptidoglycan in *Escherichia coli* is the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate by phosphoglucosamine mutase, which in *Escherichia coli* is the product of the *glmM* gene. The involvement of this enzyme activity in the pathway of lipopolysaccharide and peptidoglycan biosynthesis was recently confirmed with the cloning of the *glmM* gene. Consequently, the regulation of *glmM* gene, and its cognate product, phosphoglucosamine mutase, has not been studied in detail. It has been shown, however, that the phosphoglucosamine mutase, like all other hexosephosphate mutase enzymes studied, is regulated by phosphorylation. This type of regulation at the enzyme level

is typically exquisitely sensitive to levels of the pathway end products. Thus, carbon flow through phosphoglucosamine mutase can be self-regulating and may not be a problem as glucosamine-6-phosphate accumulates. Since the sequence of the *glmM* gene is known, however, it is a preferred embodiment of the present invention to provide a microorganism in which the gene encoding phosphoglucosamine mutase is interrupted or deleted. More preferably, the gene encoding phosphoglucosamine mutase is down-regulated, but not completely inactivated, by a mutation, so as not to completely block the biosynthesis of the critical cell envelope components.

Another pathway which results in the conversion of glucosamine-6-phosphate to another compound is catalyzed by the enzyme, *N*-acetylglucosamine-6-phosphate deacetylase. *N*-acetylglucosamine-6-phosphate deacetylase is capable of catalyzing the reverse reaction of converting glucosamine-6-phosphate (plus acetyl CoA) to *N*-acetyl-glucosamine-6-phosphate. This could result in futile cycling of glucosamine-6-phosphate and *N*-acetyl-glucosamine-6-phosphate and result in a product composed of a mixture of glucosamine and *N*-acetyl-glucosamine. Therefore, it is a further embodiment of the present invention to provide a genetically modified microorganism in which the gene encoding *N*-acetylglucosamine-6-phosphate deacetylase, which is the *nagA* gene in *Escherichia coli,* is inactivated.

It is a further embodiment of the present invention to inactivate the transport systems for glucosamine in a microorganism such that once the glucosamine is excreted by the cell it is not taken back up. This modification is helpful for avoiding a high intracellular level of glucosamine

which could be toxic to the cells, and facilitates recovery of the product, since the product remains extracellular. In a preferred embodiment of the present invention, the transportation systems for glucosamine are inactivated to keep glucosamine outside of the microorganism once it is excreted by the microorganism. During growth of *Escherichia coli* on glucosamine as sole carbon source, glucosamine is transported into the cell by the PEP:mannose phosphotransferase (PTS) system, which is not only capable of transporting glucosamine into the cell, but is also induced by glucosamine. It is therefore an embodiment of the present invention to provide a microorganism lacking the ability to transport glucosamine into the cell. For example, a *manXYZ* mutant (i.e., an *Escherichia coli* lacking or having a mutation in the genes encoding EIIM,P/III^{Man} of the PEP:mannose PTS) can not transport glucosamine into the cell by this mechanism. The PEP:glucose PTS of *Escherichia coli*, on the other hand, is capable of transporting both glucose and glucosamine into the cell, but glucosamine cannot induce this system. Thus, in order to grow a *manXYZ* mutant on glucosamine, the cells must first be grown on glucose to induce expression of the (alternate) glucose transport system and allow glucose (the preferred carbon source) to be transported into the cell. These induced cells are then capable of transporting glucosamine into the cell via the glucose transporter. A similar situation exists for transport of glucosamine by the PEP:fructose PTS, although in this case glucosamine transport by the enzyme II^{Fru} is poor. Methods to inhibit these secondary glucosamine transport pathways are discussed below. It is yet another embodiment of the present invention to provide a microorganism having a decreased function in the

PEP:glucose PTS (described above). Such a modification may be necessary to avoid "reabsorption of glucosamine from the culture medium. For example, a *ptsG* mutant (i.e., an *Escherichia coli* lacking or having a mutation in the genes encoding enzyme II^{Glc} of the PEP:glucose PTS). Since such microorganisms will have reduced ability to grow using glucose as a carbon source, such organisms can be further genetically modified to take up glucose by a PEP:glucose PTS-independent mechanism. It is has been shown, for example, that mutant microorganisms can be selected which are defective in the PEP:glucose PTS and still have an ability to grow on glucose (Flores et al., 1996, *Nature Biotechnology* 14:620-623).

DNA sequencing of the *nag* regulon in *Escherichia coli* reveals that the *nagE* gene, encoding the *N*-acetyl-glucosamine-specific enzyme II^{Nag} protein of the PEP:sugar phosphotransferase (PTS) system, which is involved in glucosamine transport into the cell, resides on one arm of the regulon and is transcribed divergently from the other *nag* genes *(nagBACD)* located on the other arm of the regulon. Therefore, another genetic modification that would result in decreased ability of an *Escherichia coli* to transport glucosamine into the cell is an inactivation or deletion of the *nagE* gene, or a gene encoding a similar enzyme in any microorganism used in a method of the present invention.

As discussed above, in one embodiment of the present invention, a genetically modified *Escherichia coli* microorganism useful in a method of the present invention has a deletion of the entire *nag* regulon. Deletion of the entire chromosomal *nag* regulon is preferred, because many genes which are deleterious to the production of glucosamine-6-phosphate are inactivated together. The genes, *nagA, nagB* and *nagE,*

have been discussed in detail above. The *nagC* gene encodes a regulatory protein that acts as a repressor of the *nag* regulon as well as both an activator and repressor of the *glmUS* operon. The *glm* genes are discussed in detail above. The function of the *nagD* gene is not known, but is believed to be related to amino sugar metabolism as it resides within the *nag* regulon. Thus, in *Escherichia coli*, a complete deletion of the *nag* regulon avoids catabolism of the initial intracellular product (glucosamine-6-phosphate) in a strain of *Escherichia coli* designed to overproduce glucosamine. A preferred *Escherichia coli* mutant strain having a deletion of the *nag* regulon is an *Escherichia coli* having a *ΔnagEBACD*::tc deletion/insertion.

With regard to activation of the *glmUS* operon (a function of *nagC*), although activation of the *glmS* gene, encoding glucosamine-6-phosphate synthase, is desirable, an increase in the level of the *glmU* gene product, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase could be deleterious to accumulation of glucosamine-6-phosphate as it could lead to siphoning off of carbon flow toward cell envelope components. It is therefore an embodiment of the present invention to inactivate glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase in a microorganism useful in a method of the present invention. In a microorganism in which the *glmUS* operon, or its equivalent, has been inactivated or deleted, it is a further embodiment of the present invention to genetically modify the microorganism by recombinantly producing the gene encoding glucosamine-6-phosphate synthase under control of an artificial promoter in the microorganism.

The initial intracellular product in the genetically modified microorganism described herein is glucosamine-6-phosphate. In many microorganisms, including *Escherichia coli*, glucosamine-6-phosphate is typically dephosphorylated to glucosamine prior to transport out of the cell. Nonetheless, it is yet another embodiment of the present invention to provide a microorganism which is genetically modified to have a suitable phosphatase activity for the conversion of glucosamine-6-phosphate to glucosamine. Such a phosphatase can include, but is not limited to, for example, alkaline phosphatase. In a preferred embodiment, such an *Escherichia coli* has an enhanced (i.e., increased) level of phosphatase activity (i.e., phosphatase action).

As noted above, in the method for production of glucosamine of the present invention, a microorganism having a genetically modified amino sugar metabolic pathway is cultured in a fermentation medium for production of glucosamine. An appropriate, or effective, fermentation medium refers to any medium in which a genetically modified microorganism of the present invention, when cultured, is capable of producing glucosamine. Such a medium is typically an aqueous medium comprising assimilable carbon, nitrogen and phosphate sources. Such a medium can also include appropriate salts, minerals, metals and other nutrients. One advantage of the genetic modifications to a microorganism described herein is that although such genetic modifications significantly alter the metabolism of amino sugars, they do not create any nutritional requirements for the production organism. Thus, a minimal-salts medium containing glucose as the sole carbon source is preferably used as the fermentation medium. The use of a minimal-salts-glucose medium for the glucosamine

fermentation will also facilitate recovery and purification of the glucosamine product.

Microorganisms of the present invention can be cultured in conventional fermentation bioreactors. The microorganisms can be cultured by any fermentation process which includes, but is not limited to, batch, fed-batch, cell recycle, and continuous fermentation. Preferably, microorganisms of the present invention are grown by batch or fed-batch fermentation processes.

In one embodiment of the present invention, before inoculation, the fermentation medium is brought up to the desired temperature, typically from about 20°C to about 40°C, preferably from about 25°C to about 40°C, with temperatures of from about 28°C to about 37°C, and in some embodiments, about 30°C or about 37°C being more preferred. The present inventors have discovered that glucosamine production in microorganisms of the present invention transfected with a nucleic acid molecule under control of the T7-*lac* promoter (see Examples section) continues after growth has ceased when the microorganisms are cultured at 30°C, while at 37°C, growth and glucosamine production occur in concert. Growth at 37°C is slightly better than at 30°C, but glucosamine production at 30°C is significantly better than at 37°C. It is noted that the optimum temperature for growth and glucosamine production by a microorganism of the present invention can vary according to a variety of factors. For example, the selection of a particular promoter for expression of a recombinant nucleic acid molecule in the microorganism can affect the optimum culture temperature. One of ordinary skill in the art can readily determine the optimum growth and glucosamine production temperature for any microorganism of the present

invention using standard techniques, such as those described in the Examples section for one microorganism of the present invention.

The medium is inoculated with an actively growing culture of the genetically modified microorganism in an amount sufficient to produce, after a reasonable growth period, a high cell density. The cells are grown to a cell density of at least about 10 g/l, preferably between about 10 g/l and about 40 g/l, and more preferably at least about 40 g/l. This process typically requires about 10-60 hours.

Sufficient oxygen must be added to the medium during the course of the fermentation to maintain cell growth during the initial cell growth and to maintain metabolism and glucosamine production. Oxygen is conveniently provided by agitation and aeration of the medium. Conventional methods, such as stirring or shaking, may be used to agitate and aerate the medium. Preferably the oxygen concentration in the medium is greater than about 15% of the saturation value (i.e., the solubility of oxygen in the medium at atmospheric pressure and about 30-40°C) and more preferably greater than about 20% of the saturation value, although excursions to lower concentrations may occur if fermentation is not adversely affected. The oxygen concentration of the medium can be monitored by conventional methods, such as with an oxygen electrode. Other sources of oxygen, such as undiluted oxygen gas and oxygen gas diluted with inert gas other than nitrogen, can be used.

Since the production of glucosamine by fermentation is preferably based on using glucose as the sole carbon source, in a preferred embodiment, in *Escherichia coli*, the PEP:glucose PTS will be induced. Accordingly, even in the

absence of a functional EIIM,P/III^{Man} of the PEP:mannose PTS (e.g., in an *Escherichia coli* having a *manXYZ* mutation), the product, glucosamine, will still be taken up by the cells via the induced glucose transport system. In the presence of excess glucose, however, uptake of glucosamine is severely repressed. Thus, it is one embodiment of the present invention to prevent uptake of the glucosamine product by maintaining an excess of glucose in the fermentation bioreactor. As used herein, "an excess" of glucose refers to an amount of glucose above that which is required to maintain the growth of the microorganism under normal conditions, such as the culturing conditions described above. Preferably, the glucose concentration is maintained at a concentration of from about 0.5% to about 5% weight/volume of the fermentation medium. In another embodiment, the glucose concentration is maintained at a concentration of from about 5 g/L to about 50 g/L of the fermentation medium, and even more preferably, from about 5 g/L to about 20 g/L of the fermentation medium. In one embodiment, the glucose concentration of the fermentation medium is monitored by any suitable method (e.g., by using glucose test strips), and when the glucose concentration is at or near depletion, additional glucose can be added to the medium. In another embodiment, the glucose concentration is maintained by semi-continuous or continuous feeding of the fermentation medium. The parameters disclosed herein for glucose can be applied to any carbon source used in the fermentation medium of the present invention. It is further understood that the carbon source can be allowed to reach undetectable levels for any appropriate amount of time during the fermentation if it enhances the glucosamine production process.

It is a further embodiment of the present invention to supplement and/or control other components and parameters of the fermentation medium, as necessary to maintain and/or enhance the production of glucosamine by a production organism. For example, in one embodiment, the fermentation medium includes ammonium sulfate, and the ammonium sulfate concentration in the culture medium is supplemented by the addition of excess ammonium sulfate. Preferably, the amount of ammonium sulfate is maintained at a level of from about 0.1% to about 1% (weight/volume) in the fermentation medium, and preferably, at about 0.5%. In yet another embodiment, the pH of the fermentation medium is monitored for fluctuations in pH. In the fermentation method of the present invention, the pH is preferably maintained at a pH of from about pH 6.0 to about pH 8.0, and more preferably, at about pH 7.0. In the method of the present invention, if the starting pH of the fermentation medium is pH 7.0, the pH of the fermentation medium is monitored for significant variations from pH 7.0, and is adjusted accordingly, for example, by the addition of sodium hydroxide.

A further embodiment of the present invention is to redirect carbon flux from acetate production to the production of less toxic byproducts. By such methods, problems of toxicity associated with an excess of glucose in the fermentation medium can be avoided. Methods to redirect carbon flux from acetate production are known in the art.

In a batch fermentation process of the present invention, fermentation is continued until the formation of glucosamine, as evidenced by the accumulation of extracellular glucosamine, essentially ceases. The total fermentation time is typically from about 40 to about 60 hours, and more preferably, about 48

hours. In a continuous fermentation process, glucosamine can be removed from the bioreactor as it accumulates in the medium. The method of the present invention results in production of a product which can include intracellular or extracellular glucosamine-6-phosphate and intracellular or extracellular glucosamine.

The method of the present invention further includes recovering the product, which can be intracellular glucosamine-6-phosphate or extracellular glucosamine. The phrase "recovering glucosamine" refers simply to collecting the product from the fermentation bioreactor and need not imply additional steps of separation or purification. For example, the step of recovering can refer to removing the entire culture (i.e., the microorganism and the fermentation medium) from the bioreactor, removing the fermentation medium containing extracellular glucosamine from the bioreactor, and/or removing the microorganism containing intracellular glucosamine-6-phosphate from the bioreactor. These steps can be followed by further purification steps. Glucosamine is preferably recovered in substantially pure form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the glucosamine as a nutriceutical compound for commercial sale. In one embodiment, the glucosamine product is preferably separated from the production organism and other fermentation medium constituents. Methods to accomplish such separation are described below.

Preferably, by the method of the present invention, at least about 1 g/L of product (i.e., glucosamine and/or glucosamine-6-phosphate) are recovered from the microorganism and/or fermentation medium. More preferably, by the method of

the present invention, at least about 5 g/L, and even more preferably, at least about 10 g/L, and even more preferably, at least about 20 g/L and even more preferably, at least about 50 g/L of product are recovered. In one embodiment, glucosamine product is recovered in an amount from about 1 g/L to about 50 g/L.

Typically, most of the glucosamine produced in the present process is extracellular. The microorganism can be removed from the fermentation medium by conventional methods, such as by filtration or centrifugation. In one embodiment, the step of recovering the product includes the purification of glucosamine from the fermentation medium. Glucosamine can be recovered from the cell-free fermentation medium by conventional methods, such as chromatography, extraction, crystallization (e.g., evaporative crystallization), membrane separation, reverse osmosis and distillation. In a preferred embodiment, glucosamine is recovered from the cell-free fermentation medium by crystallization. In another embodiment, the step of recovering the product includes the step of concentrating the extracellular glucosamine.

In one embodiment, glucosamine-6-phosphate accumulates intracellularly, the step of recovering the product includes isolating glucosamine-6-phosphate from the microorganism. For example, the product can be recovered by lysing the microorganism cells by a method which does not degrade the glucosamine product, centrifuging the lysate to remove insoluble cellular debris, and then recovering the glucosamine and/or glucosamine-6-phosphate product by a conventional method as described above.

The initial intracellular product in the genetically modified microorganism described herein is glucosamine-6-

phosphate. It is generally accepted that phosphorylated intermediates are dephosphorylated during export from the microorganism, most likely due to the presence of alkaline phosphatase in the periplasmic space of the microorganism. In one embodiment of the present invention, glucosamine-6-phosphate is dephosphorylated before or during export from the cell by naturally occurring phosphatases in order to facilitate the production of the desired product, glucosamine. In this embodiment, the need for amplification of a recombinantly provided phosphatase activity in the cell or treatment of the fermentation medium with a phosphatase is obviated. In another embodiment, the level of phosphatase in the production organism is increased by a method including, but not limited to, genetic modification of an endogenous phosphatase gene or by recombinant modification of the microorganism to express a phosphatase gene. In yet another embodiment, the recovered fermentation medium is treated with a phosphatase after glucosamine-6-phosphate is released into the medium, such as when cells are lysed as described above.

As noted above, the process of the present invention produces significant amounts of extracellular glucosamine. In particular, the process produces extracellular glucosamine such that greater than about 50% of total glucosamine is extracellular, more preferably greater than about 75% of total glucosamine is extracellular, and most preferably greater than about 90% of total glucosamine is extracellular. By the method of the present invention, production of an extracellular glucosamine concentration can be achieved which is greater than about 1 g/l, more preferably greater than about 5 g/l, even more preferably greater than about 10 g/l,

and even more preferably greater than about 20 g/L and even more preferably greater than about 50 g/l.

One embodiment of the present invention relates to a method to produce glucosamine by fermentation which includes the steps of (a) culturing an *Escherichia coli* having a genetically modified amino sugar metabolic pathway in a fermentation medium comprising assimilable sources of carbon, nitrogen and phosphate to produce a product, and (b) recovering the product. The product includes intracellular glucosamine-6-phosphate which is recovered from the *Escherichia coli* and/or extracellular glucosamine which is recovered from the fermentation medium.

One embodiment of the present invention relates to a microorganism for producing glucosamine by a biosynthetic process. The microorganism is transformed with a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase operatively linked to a transcription control sequence. The recombinant nucleic acid molecule has a genetic modification which reduces glucosamine-6-phosphate product inhibition of the glucosamine-6-phosphate synthase. Expression of the recombinant nucleic acid molecule increases expression of the glucosamine-6-phosphate synthase by the microorganism. In a preferred embodiment, the recombinant nucleic acid molecule is integrated into the genome of the microorganism. In a further embodiment, the microorganism has at least one additional genetic modification in a gene encoding a protein selected from the group of *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, *N*-acetyl-glucosamine-specific enzyme II^{Nag}, phosphoglucosamine mutase, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate

uridyltransferase, phosphofructokinase, Enzyme II^{Glc} of the PEP:glucose PTS, EIIM,P/III^{Man} of the PEP:mannose PTS, and/or a phosphatase. The genetic modification decreases the action of the protein, except in the case of the phosphatase, in which the action of the phosphatase is preferably increased. In another preferred embodiment, the microorganism has a modification in genes encoding *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and *N*-acetyl-glucosamine-specific enzyme II^{Nag}, wherein the genetic modification decreases action of the protein. In one embodiment, the genetic modification is a deletion of at least a portion of the genes.

In a preferred embodiment, the genetically modified microorganism is a bacterium or a yeast, and more preferably, a bacterium of the genus *Escherichia*, and even more preferably, *Escherichia coli*. A genetically modified *Escherichia coli* preferably has a modification in a gene which includes, but is not limited to, *nagA*, *nagB*, *nagC*, *nagD*, *nagE*, *manXYZ*, *glmM*, *pfkB*, *pfkA*, *glmU*, *glmS*, *ptsG* or a phosphatase gene. In another embodiment, such a genetically modified *Escherichia coli* has a deletion of *nag* regulon genes, and in yet another embodiment, a deletion of *nag* regulon genes and a genetic modification in *manXYZ* genes such that the proteins encoded by the *manXYZ* genes have decreased action.

Yet another embodiment of the present invention relates to a microorganism for producing glucosamine by a biosynthetic process which has a recombinant nucleic acid molecule encoding glucosamine-6-phosphate synthase operatively linked to a transcription control sequence; and at least one genetic modification in a gene encoding a protein selected from the group of *N*-acetylglucosamine-6-phosphate deacetylase,

glucosamine-6-phosphate deaminase, *N*-acetyl-glucosamine-specific enzyme II^{Nag}, phosphoglucosamine mutase, glucosamine-1-phosphate acetyltransferase-*N*-acetylglucosamine-1-phosphate uridyltransferase, phosphofructokinase, Enzyme II^{Glc} of the PEP:glucose PTS, and/or EIIM,P/III^{Man} of the PEP:mannose PTS. The genetic modification decreases action of said protein and expression of the recombinant nucleic acid molecule increases expression of the glucosamine-6-phosphate synthase by the microorganism. In another embodiment, the microorganism has at least one genetic modification in a phosphatase gene, such that the phosphatase encoded by such gene has increased action. In a preferred embodiment, the recombinant nucleic acid molecule is integrated into the genome of the microorganism.

Another embodiment of the present invention relates to any of the above-described microorganisms which produces at least about 1 g/L of glucosamine when cultured for about 24 hours at 37°C to a cell density of at least about 8 g/L by dry cell weight, in a pH 7.0 fermentation medium comprising: 14 g/L K_2HPO_4, 16 g/L KH_2PO_4, 1 g/L $Na_3Citrate{\cdot}2H_2O$, 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose, 10 mM $MgSO_4$, 1 mM $CaCl_2$, and 1 mM IPTG.

A more preferred embodiment of the present invention relates to any of the above-described microorganisms which produces at least about 1 g/L of glucosamine when cultured for about 10 to about 60 hours at from about 28°C to about 37°C to a cell density of at least about 8 g/L by dry cell weight, in a pH 7.0 fermentation medium comprising: 14 g/L K_2HPO_4, 16 g/L KH_2PO_4, 1 g/L $Na_3Citrate{\cdot}2H_2O$, 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose, 10 mM $MgSO_4$, 1 mM $CaCl_2$, and from about 0.2mM to about 1 mM IPTG. In a preferred embodiment, the amount of IPTG is about 0.2mM.

Yet another embodiment of the present invention relates to any of the above-described genetically modified microorganisms which produce at least about 1 g/L, and preferably at least about 5 g/L, and more preferably, at least about 10 g/L, and even more preferably, at least about 20 g/L, and even more preferably, at least about 50 g/L of glucosamine and/or glucosamine-6-phosphate when cultured under the culture conditions as described herein. Another embodiment of the present invention relates to any of the above-described genetically modified microorganisms which produce at least about 2-fold more glucosamine and/or glucosamine-6-phosphate, and preferably at least about 5-fold, and more preferably at least about 10-fold, and more preferably at least about 25-fold, and more preferably at least about 50-fold, and even more preferably at least about 100-fold, and even more preferably, at least about 200-fold more glucosamine and/or glucosamine-6-phosphate synthase than a wild-type (i.e., non-modified, naturally occurring) microorganism cultured under the same conditions as the genetically modified microorganism.

A number of specific microorganisms are identified in the Examples section. Additional embodiments of the present invention include these microorganisms and microorganisms having the identifying characteristics of the microorganisms specifically identified in the Examples. Such microorganisms are preferably yeast or bacteria, more preferably, are bacteria, and most preferably are *E. coli*. Such identifying characteristics can include any or all genotypic and/or phenotypic characteristics of the microorganisms in the Examples, including their abilities to produce glucosamine.

Preferred microorganisms of the present invention include strains of *Escherichia coli* which have been transformed with a recombinant nucleic acid molecule encoding glucosamine-6-

phosphate synthase. Preferably, such a nucleic acid molecule is integrated into the genome of the microorganism. A particularly preferred microorganism is *Escherichia coli* strain 2123-12. Strain 2123-12 has integrated into its genome a recombinant nucleic acid molecule comprising a nucleic acid sequence SEQ ID NO:15, which represents the coding region of a wild-type (i.e., normal, unmodified, or naturally occurring) glucosamine-6-phosphate synthase enzyme having amino acid sequence SEQ ID NO:16. Particularly preferred microorganisms of the present invention have been transformed with a nucleic acid molecule comprising a nucleic acid sequence encoding a glucosamine-6-phosphate synthase that has been genetically modified such that the synthase has increased action (described above). Most preferably, such genetic modification enhances the ability of the microorganism to produce glucosamine as compared to a microorganism which has not been transformed with such a nucleic acid molecule. Particularly preferred genetically modified microorganisms of the present invention are described in the Examples section, and include *E. coli* strains 2123-49, 2123-54, 2123-124, 2123-149 and 2123-151.

Development of a microorganism with enhanced ability to produce glucosamine by genetic modification can be accomplished using both classical strain development and molecular genetic techniques. In general, the strategy for creating a microorganism with enhanced glucosamine production is to (1) inactivate or delete at least one, and preferably more than one of the amino sugar metabolic pathways in which production of glucosamine-6-phosphate is negatively affected (e.g., inhibited), and (2) amplify at least one, and preferably more than one of the amino sugar metabolic pathways in which glucosamine-6-phosphate production is enhanced. As

such, genetically modified microorganisms of the present invention have a (a) reduced ability to convert glucosamine-6-phosphate into other compounds (i.e., inhibition of glucosamine-6-phosphate catabolic or anabolic pathways), (b) an enhanced ability to produce (i.e., synthesize) glucosamine-6-phosphate, (c) a reduced ability to transport glucosamine into the cell, (d) an enhanced ability to transport glucosamine-6-phosphate or glucosamine out of the cell, and/or (e) a reduced ability to use substrates involved in the production of glucosamine-6-P for competing biochemical reactions.

As previously discussed herein, in one embodiment, a genetically modified microorganism can be a microorganism in which nucleic acid molecules have been deleted, inserted or modified, such as by insertion, deletion, substitution, and/or inversion of nucleotides, in such a manner that such modifications provide the desired effect within the microorganism. Such genetic modifications can, in some embodiments, be within the coding region for a protein encoded by the nucleic acid molecule which results in amino acid modifications such as insertions, deletions, substitutions in the amino acid sequence of the protein which provide the desired effect within the microorganisms. A genetically modified microorganism can be modified by recombinant technology, such as by introduction of an isolated nucleic acid molecule into a microorganism. For example, a genetically modified microorganism can be transfected with a recombinant nucleic acid molecule encoding a protein of interest, such as a protein for which increased expression is desired. The transfected nucleic acid molecule can remain extrachromosomal or can integrate into one or more sites

within a chromosome of the transfected (i.e., recombinant) host cell in such a manner that its ability to be expressed is retained. Preferably, once a host cell of the present invention is transfected with a nucleic acid molecule, the nucleic acid molecule is integrated into the host cell genome. A significant advantage of integration is that the nucleic acid molecule is stably maintained in the cell. In a preferred embodiment, the integrated nucleic acid molecule is operatively linked to a transcription control sequence (described below) which can be induced to control expression of the nucleic acid molecule.

A nucleic acid molecule can be integrated into the genome of the host cell either by random or targeted integration. Such methods of integration are known in the art. For example, as described in detail in Example 2, *E. coli* strain ATCC 47002 (Table 1) contains mutations that confer upon it an inability to maintain plasmids which contain a ColE1 origin of replication. When such plasmids are transferred to this strain, selection for genetic markers contained on the plasmid results in integration of the plasmid into the chromosome. This strain can be transformed, for example, with plasmids containing the gene of interest and a selectable marker flanked by the 5'- and 3'-termini of the *E. coli lacZ* gene. The *lacZ* sequences target the incoming DNA to the *lacZ* gene contained in the chromosome. Integration at the *lacZ* locus replaces the intact *lacZ* gene, which encodes the enzyme β-galactosidase, with a partial *lacZ* gene interrupted by the gene of interest. Successful integrants can be selected for β-galactosidase negativity. A genetically modified microorganism can also be produced by introducing nucleic acid molecules into a recipient cell genome by a method such as by

using a transducing bacteriophage. The use of recombinant technology and transducing bacteriophage technology to produce several different genetically modified microorganism of the present invention is known in the art and is described in detail in the Examples section. According to the present invention, a gene, for example the *pstG* gene, includes all nucleic acid sequences related to a natural *pstG* gene such as regulatory regions that control production of the *pstG* protein (Enzyme II^{Glc} of the PEP:glucose PTS) encoded by that gene (such as, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself. In another embodiment, a gene, for example the *pstG* gene, can be an allelic variant (i.e., a naturally occurring allelic variant) that includes a similar but not identical sequence to the nucleic acid sequence encoding a given *pstG* gene. An allelic variant of a *pstG* gene which has a given nucleic acid sequence is a gene that occurs at essentially the same locus (or loci) in the genome as the gene having the given nucleic acid sequence, but which, due to natural variations caused by, for example, mutation or recombination, has a similar but not identical sequence. Allelic variants typically encode proteins having similar activity to that of the protein encoded by the gene to which they are being compared. Allelic variants can also comprise alterations in the 5' or 3' untranslated regions of the gene (e.g., in regulatory control regions). Allelic variants are well known to those skilled in the art and would be expected to be found within a given microorganism, such as an *E. coli*, and/or among a group of two or more microorganisms.

Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase

"nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a gene involved in an amino sugar metabolic pathway. In addition, the phrase "recombinant molecule" primarily refers to a nucleic acid molecule operatively linked to a transcription control sequence, but can be used interchangeably with the phrase "nucleic acid molecule" which is isolated and expressed in a host cell.

Knowing the nucleic acid sequences of certain nucleic acid molecules of the present invention, and particularly *Escherichia coli* nucleic acid molecules, allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules and/or (b) obtain nucleic acid molecules including at least a portion of such nucleic acid molecules (e.g., nucleic acid molecules including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions). Such nucleic acid molecules can be obtained in a variety of ways including traditional cloning techniques using oligonucleotide probes of to screen appropriate libraries or DNA and PCR amplification of appropriate libraries or DNA using oligonucleotide primers. Preferred libraries to screen or from which to amplify nucleic acid molecule include bacterial and yeast genomic DNA libraries, and in particular, *Escherichia coli* genomic DNA libraries. Techniques to clone and amplify genes are disclosed, for example, in Sambrook et al., *ibid.*

In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not reflect the extent to which the nucleic acid molecule has been

purified. An isolated nucleic acid molecule can include DNA, RNA, or derivatives of either DNA or RNA. There is no limit, other than a practical limit, on the maximal size of a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof.

An isolated nucleic acid molecule of the present invention can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. An isolated nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated nucleic acid molecules include natural nucleic acid molecules and homologues thereof, including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications provide the desired effect within the microorganism.

A nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, for example, Sambrook et al., *ibid.*). For example, nucleic acid molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, PCR amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic

acid molecules and combinations thereof. Nucleic acid molecule homologues can be selected from a mixture of modified nucleic acids by screening for the function of the protein encoded by the nucleic acid and/or by hybridization with a wild-type gene. Examples of such techniques are described in detail in the Examples section.

In one embodiment of the present invention, a nucleic acid homologue of a nucleic acid molecule of the present invention preferably comprises a genetic modification which results in an modification of the action of the protein encoded by the nucleic acid homologue. For example, in one embodiment of the present invention, a genetically modified recombinant nucleic acid molecule is provided which comprises a nucleic acid sequence encoding a glucosamine-6-phosphate synthase protein homologue, wherein the genetic modification increases the action of the glucosamine-6-phosphate synthase homologue, preferably as compared to a recombinant nucleic acid molecule encoding a naturally occurring glucosamine-6-phosphate synthase in the absence of such genetic modification. Such a genetic modification can increase the action of the glucosamine-6-phosphate synthase, for example, by encoding a glucosamine-6-phosphate synthase having reduced glucosamine-6-phosphate product inhibition and/or increased specific activity. Such recombinant nucleic acid molecules having genetic modifications are referred to herein as nucleic acid homologues of wild-type nucleic acid molecules encoding glucosamine-6-phosphate synthase. According to the present invention, proteins having modifications as a result of genetic modifications in the nucleic acid molecules encoding the proteins are referred to herein as protein homologues, or homologues of the given protein.

Accordingly, a glucosamine-6-phosphate synthase protein, for example, which has glucosamine-6-phosphate synthase

activity and is useful in the present invention, can be a full-length glucosamine-6-phosphate synthase protein, an enzymatically active portion of a full-length glucosamine-6-phosphate synthase protein, or any homologue of such proteins, such as a glucosamine-6-phosphate synthase protein having at least one or a few amino acid modifications in which amino acid residues have been deleted (e.g., a truncated version of the protein, such as a peptide), inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation and/or addition of glycosylphosphatidyl inositol).

A protein homologue of any of the proteins within the amino sugar metabolic pathways as described in the present invention is a protein having an amino acid sequence that is sufficiently similar to a natural protein amino acid sequence (i.e., naturally occurring, unmodified, or wild-type) that a nucleic acid sequence encoding the homologue is capable of hybridizing under stringent conditions to (i.e., with) a nucleic acid molecule encoding the natural protein (i.e., to the complement of the nucleic acid strand encoding the natural protein amino acid sequence). A nucleic acid sequence complement of any nucleic acid sequence of the present invention refers to the nucleic acid sequence of the nucleic acid strand that is complementary to (i.e., can form a double helix with the entire molecule) the strand for which the sequence is cited. It is to be noted that a double-stranded nucleic acid molecule of the present invention for which a nucleic acid sequence has been determined for one strand that is represented by a SEQ ID NO also comprises a complementary strand having a sequence that is a complement of that SEQ ID NO. As such, nucleic acid molecules of the present invention, which can be either double-stranded or single-stranded,

include those nucleic acid molecules that form stable hybrids under stringent hybridization conditions with either a given SEQ ID NO denoted herein and/or with the complement of that SEQ ID NO, which may or may not be denoted herein. Methods to deduce a complementary sequence are known to those skilled in the art. The minimal size of a protein homologue of the present invention is a size sufficient to be encoded by a nucleic acid molecule capable of forming a stable hybrid with the complementary sequence of a nucleic acid molecule encoding the corresponding natural protein. Additionally, the minimal size of a protein homologue of the present invention is a size sufficient to have glucosamine-6-phosphate synthase action (e.g., a catalytically or enzymatically active portion), and preferably, increased glucosamine-6-phosphate synthase action. As such, the size of the nucleic acid molecule encoding such a protein homologue is dependent on nucleic acid composition and percent homology between the nucleic acid molecule and complementary sequence as well as upon hybridization conditions per se (e.g., temperature, salt concentration, and formamide concentration). There is no limit, other than a practical limit, on the maximal size of such a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof. Similarly, the minimal size of a protein homologue of the present invention is from about 4 to about 6 amino acids in length, with preferred sizes depending on whether a full-length, multivalent (i.e., fusion protein having more than one domain each of which has a function), or functional portions of such proteins are desired.

As used herein, stringent hybridization conditions refer to standard hybridization conditions under which nucleic acid molecules are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in

Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety (see specifically, pages 9.31-9.62, 11.7 and 11.45-11.61). In addition, formulae to calculate the appropriate hybridization and wash conditions to achieve hybridization permitting varying degrees of mismatch of nucleotides are disclosed, for example, in Meinkoth et al., 1984, *Anal. Biochem.* 138, 267-284; Meinkoth et al., *ibid.*, is incorporated by reference herein in its entirety.

More particularly, stringent hybridization conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 70% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction, more particularly at least about 75%, and most particularly at least about 80%. Such conditions will vary, depending on whether DNA:RNA or DNA:DNA hybrids are being formed. Calculated melting temperatures for DNA:DNA hybrids are 10°C less than for DNA:RNA hybrids. In particular embodiments, stringent hybridization conditions for DNA:DNA hybrids include hybridization at an ionic strength of 0.1X SSC (0.157 M Na^+) at a temperature of between about 20°C and about 35°C, more preferably, between about 28°C and about 40°C, and even more preferably, between about 35°C and about 45°C. In particular embodiments, stringent hybridization conditions for DNA:RNA hybrids include hybridization at an ionic strength of 0.1X SSC (0.157 M Na^+) at a temperature of between about 30°C and about 45°C, more preferably, between about 38°C and about 50°C, and even more preferably, between about 45°C and about 55°C. These values are based on calculations of a melting temperature for molecules larger than about 100 nucleotides, 0% formamide and a G + C content of about 50%. Alternatively,

T_m can be calculated empirically as set forth in Sambrook et al., *supra*, pages 11.55 to 11.57.

Protein homologues of proteins involved in an amino sugar metabolic pathway according to the present invention can be the result of natural allelic variation or natural mutation. Protein homologues of the present invention can also be produced using techniques known in the art including, but not limited to, direct modifications to the protein or modifications to the gene encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis, as discussed above.

In one embodiment of the present invention, a genetic modification in a recombinant nucleic acid molecule of the present invention which encodes a glucosamine-6-phosphate synthase results in at least one amino acid modification (i.e., modification in the amino acid sequence of the encoded protein) selected from the group of an addition, substitution, deletion, and/or derivatization of an amino acid residue of the glucosamine-6-phosphate synthase. Such a modification in the amino acid sequence of the encoded protein can be determined as compared to a wild-type, or naturally occurring glucosamine-6-phosphate synthase, such as a glucosamine-6-phosphate synthase having an amino acid sequence SEQ ID NO:16. One or more of such amino acid modifications results in increased action of glucosamine-6-phosphate synthase as compared to the naturally occurring glucosamine-6-phosphate synthase having amino acid sequence SEQ ID NO:16. In one embodiment, such an amino acid modification is in an amino acid sequence position in the modified protein (i.e., homologue) which corresponds to one or more of the following amino acid positions in amino acid sequence SEQ ID NO:16:

Ile(4), Ile(272), Ser(450), Ala(39), Arg(250), Gly(472), Leu(469).

In another embodiment, such an amino acid modification is selected from the group of a substitution of: (a) an amino acid residue having an aliphatic hydroxyl side group for Ile(4); (b) an amino acid residue having an aliphatic hydroxyl side group for Ile(272); (c) an amino acid residue having an aliphatic side group for Ser(450); (d) an amino acid residue having an aliphatic hydroxyl side group for Ala(39); (e) an amino acid residue having a sulfur-containing side group for Arg(250); (f) an amino acid residue having an aliphatic hydroxyl side group for Gly(472); (g) an amino acid residue having an aliphatic side group for Leu(469); and, (h) combinations of (a)-(g). According to the present invention, amino acid residues having an aliphatic hydroxyl group include serine and threonine, and amino acid residues having aliphatic side groups include glycine, alanine, valine, leucine, isoleucine and proline.

In yet another embodiment of the present invention, an amino acid modification as described above is preferably a substitution selected from the group of: Ile(4) to Thr, Ile(272) to Thr, Ser(450) to Pro, Ala(39) to Thr, Arg(250) to Cys, Gly(472) to Ser, Leu(469) to Pro, and combinations thereof. Specific examples of recombinant nucleic acid molecules having genetic modifications resulting in such amino acid modifications are described in detail in the Examples section.

Preferred genetically modified recombinant nucleic acid molecules comprising a nucleic acid sequence encoding a glucosamine-6-phosphate synthase having increased action include recombinant nucleic acid molecules comprising a nucleic acid sequence which encodes a glucosamine-6-phosphate

synthase comprising an amino acid sequence selected from the group of SEQ ID NO:19, SEQ ID NO:22, SEQ ID NO:25, SEQ ID NO:28 and/or SEQ ID NO:31. Other preferred genetically modified recombinant nucleic acid molecules comprise a nucleic acid sequence selected from the group of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:29 and/or SEQ ID NO:30. Particularly preferred genetically modified recombinant nucleic acid molecules useful in the present invention include nucleic acid molecules comprising nucleic acid molecules selected from the group of pKLN23-49, pKLN23-54, pKLN23-124, pKLN23-149, pKLN23-151, nglmS-49_{2184}, nglmS-49_{1830}, nglmS-54_{2184}, nglmS-54_{1830}, nglmS-124_{2184}, nglmS-124_{1830}, nglmS-149_{2184}, nglmS-149_{1630}, nglmS-151_{2184} and nglmS-151_{1830}.

The present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a bacterial cell. Such a vector can contain bacterial nucleic acid sequences that are not naturally found adjacent to the isolated nucleic acid molecules to be inserted into the vector. The vector can be either RNA or DNA and typically is a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of nucleic acid molecules. One type of recombinant vector, referred to herein as a recombinant nucleic acid molecule and described in more detail below, can be used in the expression of nucleic acid molecules. Preferred recombinant vectors are capable of replicating in a transformed bacterial or yeast cell, and in particular, in an *Escherichia coli* cell.

Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation and microinjection.

Recombinant molecules of the present invention, which can be either DNA or RNA, can also contain additional regulatory sequences, such as translation regulatory sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell. One or more recombinant molecules of the present invention can be used to produce an encoded product (e.g., a glucosamine-6-phosphate synthase). In one embodiment, an encoded product is produced by expressing a nucleic acid molecule of the present invention under conditions effective to produce the protein. Such conditions (i.e., culture conditions) have been described above and are further discussed in the Examples section. A preferred method to produce an encoded protein is by transfecting a host cell with one or more recombinant molecules of the present invention to form a recombinant cell.

As discussed above, preferred recombinant molecules of the present invention include, nglmS-28_{2184}, nglmS-28_{1830}, nglmS-49_{2184}, nglmS-49_{1830}, nglmS-54_{2184}, nglmS-54_{1830}, nglmS-124_{2184}, nglmS-124_{1830}, nglmS-149_{2184}, nglmS-149_{1830}, nglmS-151_{2184}, nglmS-151_{1830}, pKLN23-28, pKLN23-49, pKLN23-54, pKLN23-124, pKLN23-149 and/or pKLN23-151.

A recombinant cell is preferably produced by transforming a bacterial cell (i.e., a host cell) with one or more recombinant molecules, each comprising one or more nucleic acid molecules operatively linked to an expression vector containing one or more transcription control sequences. The phrase, operatively linked, refers to insertion of a nucleic

acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. In the present invention, expression vectors are typically plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in a yeast host cell or a bacterial host cell, preferably an *Escherichia coli* host cell. Preferred recombinant cells of the present invention are set forth in the Examples section.

Nucleic acid molecules of the present invention can be operatively linked to expression vectors containing regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in yeast or bacterial cells and preferably, *Escherichia coli*. A variety of such transcription control sequences are known to those skilled in the art.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into the host cell chromosome, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals, modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein. Such modifications are described in detail in the Examples section.

The following experimental results are provided for the purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

The following example describes the production of mutant *Escherichia coli* strains which are blocked in amino acid sugar metabolic pathways involving degradation of glucosamine.

The starting strain for the construction of all glucosamine overproducing strains described herein was *E. coli* W3110 (publicly available from the American Type Culture Collection as ATCC No. 25947), a prototrophic, F^- λ^- derivative of *E. coli* K-12 (Bachmann, 1987, "*Escherichia coli* and *Salmonella typhimurium*", Cellular and Molecular Biology, pp.1190-1219; incorporated herein by reference in its entirety). A list of all strains used and produced in the following examples is provided in Table 1.

Table 1. Bacterial strains.

Strain	Alias	Genotype	Source/Reference
W3110		F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻	ATCC
IBPC 522		*thi-1 argG6 argE3 his-4 mtl-1 xyl-5 rpsL tsx-29? ΔlacX74 manXYZ8 nagE47 ptsG22 zcf-229*::*Tn*10	J. Plumbridge
IBPC 566		*thi-1 argG6 argE3 his-4 mtl-1 xyl-5 rpsL tsx-29? ΔlacX74 manXYZ8 zdj-225*::*Tn*10	J. Plumbridge
IBPC 590		*thi-1 argG6 argE3 his-4 mtl-1 xyl-5 rpsL tsx-29? ΔlacX74 Δnag*::TcR	J. Plumbridge
7101-6	W3110 ptsM	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10	W3110 x $P1_{vir}$(IBPC566)
7101-7	W3110 ptsM	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10	W3110 x $P1_{vir}$(IBPC566)
7101-9	W3110 Δnag	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *Δnag*::TcR	W3110 x $P1_{vir}$(IBPC590)
7101-13	W3110 ptsM TcS	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10? TcS	7101-6 selected on TCS medium
7101-14	W3110 ptsM TcS	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10? TcS	7101-7 selected on TCS medium
7101-15	W3110 ptsM ptsG	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10? *ptsG22 zcf-229*::*Tn*10	7101-14 x $P1_{vir}$(IBPC522)
7101-17	W3110 ptsM Δnag	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10? TcS *Δnag*::TcR	7101-13 x $P1_{vir}$(IBPC590)
7101-22	W3110 ptsM ptsG TcS	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10? *ptsG22 zcf-229*::*Tn*10? TcS	7101-15 selected on TCS medium
2123-4	W3110 ptsM ptsG Δnag	F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λ⁻ *manXYZ8 zdj-225*::*Tn*10? *ptsG22 zcf-229*::*Tn*10? TcS Δnag::TcR	7101-22 x $P1_{vir}$(IBPC590)
W3110(DE3)		F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λDE3	W3110 lysogenized with λDE3
7101-9(DE3)		F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λDE3 *Δnag*::TcR	7101-9 lysogenized with λDE3
7101-17(DE3)		F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λDE3 *manXYZ8 zdj-225*::Tn10? TcS *Δnag*::TcR	7101-17 lysogenized with λDE3
2123-4(DE3)		F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)*1* λDE3 *manXYZ8 zdj-225*::Tn10? *ptsG22 zcf-229*::*Tn*10 TcS *Δnag*::TcR	2123-4 lysogenized with λDE3
BL21(DE3)		F⁻ *ompT* $hsdS_B$ *gal dcm* λDE3	Novagen, Inc.
ATCC 47002	JC7623	F⁻ *recB21 recC22 sbcB15 leu-6 ara-14 his-4* λ⁻	ATCC
T-71		F⁻ *recB21 recC22 sbcB15 leu-6 era-14 his-4* λ⁻ *lacZ*::pT7-*glmS*-Cm8H7	Integration of pT7-*glmS*-Cm into *lacZ* of ATCC47002 by transformation with pKLN23-28
T-81		F⁻ *recB21 recC22 sbcB15 leu-6 ara-14 his-4* λ⁻ *lacZ*::pT7-*glmS*-Cm8H8	Integration of pT7-*glmS*-Cm into *lacZ* of ATCC47002 by transformation with pKLN23-28
2123-5		W3110(DE3) *lacZ*::pT7-*glmS*-Cm8H7	W3110(DE3) x $P1_{vir}$(T-71)

Strain	Alias	Genotype	Source/Reference
2123-6		W3110(DE3) *lacZ*::pT7-*glmS*-Cm8H8	W3110(DE3) x $P1_{vir}$(T-81)
2123-7		W3110(DE3) *lacZ*::pT7-*glmS*-Cm8H7	W3110(DE3) x $P1_{vir}$(T-71)
2123-8		W3110(DE3) *lacZ*::pT7-*glmS*-Cm8H8	W3110(DE3) x $P1_{vir}$(T-81)
2123-9		7101-9(DE3) *lacZ*.:pT7-*glmS*-Cm8H7	7101-9(DE3)x $P1_{vir}$(T-71)
2123-10		7101-9(DE3) *lacZ*::pT7-*glmS*-Cm8H8	7101-9(DE3)x $P1_{vir}$(T-81)
2123-11		7101-17(DE3) *lacZ*::pT7-*glmS*-Cm8H7	7101-17(DE3)x $P1_{vir}$(T-71)
2123-12		7101-17(DE3) *lacZ*::pT7-*glmS*-Cm8H8	7101-17(DE3)x $P1_{vir}$(T-81)
2123-13		2123-4(DE3) *lacZ*::pT7-*glmS*-Cm8H7	2123-4(DE3)x$P1_{vir}$(T-71)
2123-14		2123-4(DE3) *lacZ*::pT7-*glmS*-Cm8H8	2123-4(DE3)x$P1_{vir}$(T-81)
NovaBlue		*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*::*Tn*10]	Novagen
LE392		F$^-$ e14$^-$ (McrA$^-$) *hsdR514*(r$^-$m$^+$) *supE44 supF58 lacY1 or Δlac(IZY)6 galK2 galT22 metB1 trpR55*	Lab collection
2123-16		LE392 *glmS13*	NG mutagenesis of LE392
2123-49		7101-17(DE3) *lacZ*::pT7-*glmS*11C-Cm8H8	Error-prone PCR with pKLN23-28; integration of mutant *glmS* into ATCC47002; transfer to 7101-17(DE3) by P1 transduction
2123-51		7101-17(DE3) *lacZ*::pT7-*glmS*52B-Cm8H8	Error-prone PCR with pKLN23-28; integration of mutant *glmS* into ATCC47002; transfer to 7101-17(DE3) by P1 transduction
2123-54		7101-17(DE3) *lacZ*::pT7-*glmS*8A-Cm8H8	Error-prone PCR with pKLN23-28; integration of mutant *glmS* into ATCC47002; transfer to 7101-17(DE3) by P1 transduction
2123-124		7101-17(DE3) *lacZ*::pT7-*glmS*94A S-Cm8H8	Error-prone PCR with pKLN23-28; integration of mutant *glmS* into ATCC47002; transfer to 7107-17(DE3) by P1 transduction
2123-149		7101-17(DE3) *lacZ*::pT7-*glmS*149-Cm8H8	pKLN23-54 *Eco*RI-*Hind*III (1.0 kb) x pKLN23-28 *Eco*RI-*Hind*III (6.4 kb); integration of mutant *glmS* into ATCC47002; transfer to 7101-17(DE3) by P1 transduction
2123-151		7101-17(DE3) *lacZ*::pT7-*glmS*151-Cm8H8	pKLN23-54 *Eco*RI-*Hind*III (1.0 kb) x pKLN23-28 *Eco*RI-*Hind*III (6 4 kb); integration of mutant *glmS* into ATCC47002; transfer to 7101-17(DE3) by P1 transduction

Host strains blocked for glucosamine uptake and degradation were constructed by introducing mutations in the *nagE*, *manXYZ* and *ptsG* genes, which block transport of

glucosamine, and the *nagA, -B, -C,* and *-D* genes, which are involved in metabolism of glucosamine-6-phosphate. Each of these genes has been described in detail previously herein. Mutations in these genes were introduced into strains using the transducing bacteriophage $P1_{vir}$ (as described in Miller, 1972, "Experiments in Molecular Genetics", Cold Spring Harbor Laboratory, which is incorporated herein by reference in its entirety).

In this technique, genes or mutations from one strain (the donor strain) are transferred to a recipient strain using the bacteriophage $P1_{vir}$. When bacteriophage $P1_{vir}$ is grown on the donor strain, a small portion of the phage particles that are produced contain chromosomal DNA from the donor rather than the normal complement of phage DNA. Upon infection of the recipient strain with bacteriophage grown on the donor strain, those bacteriophage particles containing chromosomal DNA from the donor strain can transfer that DNA to the recipient strain. If there is a strong selection for the DNA from the donor strain, recipient strains containing the appropriate gene or mutation from the donor strain can be selected.

To grow $P1_{vir}$ on a donor strain, an existing bacteriophage stock was used to infect a culture of that strain. The recipient strain was grown at 37°C in LBMC medium (10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$) until the absorbance at 600 nm was approximately 1.0, corresponding to approximately 6 x 10^8 cells per mL of culture. One mL of the culture was then infected with a dilution of the phage stock at a ratio of approximately one phage per 10 cells. The mixture was incubated without shaking for 20 minutes at 37°C, then transferred to 10 mL of prewarmed

LBMC broth in a 125 mL baffled Erlenmeyer flask. The resulting culture was shaken vigorously for 2-3 hours at 37°C. During this period, it was generally observed that the culture would become more turbid, indicating bacterial growth. Toward the end of this incubation period, the culture would become clear, indicating cell lysis due to bacteriophage growth. After lysis had occurred, the culture was cooled on ice, a few drops of chloroform were added, and the flask was shaken briefly. The contents of the flask were then centrifuged to remove the cell debris and chloroform, and the resulting supernatant generally contained between 10^8 and 10^9 bacteriophage per mL.

Mutations were transferred to recipient strains by transduction with $P1_{vir}$ grown on the appropriate donor strain as described above. For transduction with $P1_{vir}$, a culture of the recipient strain was grown overnight at 37°C in LBMC broth. 0.1 mL of culture was mixed with 0.1 mL of bacteriophage lysate or a serial dilution of the lysate in a sterile test tube and incubated at 37°C for 20 minutes. 0.2 mL of 1 M sodium citrate was added to the tube, and the mixture was plated to selective medium. For each transduction, controls containing uninfected cells and bacteriophage lysates without cells were performed as described above. For the production of strains blocked in glucosamine degradation, selections were for tetracycline resistance as described below. Tetracycline resistant mutants were selected by plating to LB medium (10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 12.5 µg/mL tetracycline and 10 mM sodium citrate.

The mutations in the *nag* genes were introduced simultaneously as a deletion mutation ($\Delta nag::Tc^R$). In strain

IBPC590 (Plumbridge, Table 1), which contains this mutation, the *nag* genes have been replaced by a tetracycline-resistance (Tc^R) determinant. As a result, the mutation which removes the *nag* functions was transferred to appropriate recipient hosts by selection for tetracycline resistance. In this case, since the Tc^R determinant was contained within the mutation of interest, the Δ*nag* and Tc^R mutations were 100% linked. That is, all of the recipient strains receiving the Tc^R determinant from IBPC590 also received the Δ*nag* mutation. This was confirmed by examining the growth phenotype of the tetracycline resistant strains resulting from infection with $P1_{vir}$ grown on IBPC590. All such strains were unable to grow on media containing glucosamine or N-acetylglucosamine as carbon sources, indicating the presence of the Δ*nag* mutation.

Mutations in the *manXYZ* and *ptsG* genes were also introduced by $P1_{vir}$ transduction using phage grown on strains IBPC566 and IBPC522 (Plumbridge, Table 1), respectively. These strains also contained tetracycline-resistance determinants linked to the mutations of interest (designated *zdj-225*::*Tn*10 and *zcf-229*::*Tn*10, respectively). In these strains, the Tc^R determinants were not within the gene but were linked to the gene. Accordingly, not all recipient strains receiving the Tc^R determinant contained the mutations of interest. The degree of linkage is indicative of the distance on the chromosome between the Tc^R determinant and the mutation of interest. As a result, it was necessary to screen tetracycline resistant strains for *manXYZ* and *ptsG*. The *manXYZ* strains grew slowly on mannose and failed to grow on glucosamine as sole carbon sources for growth. The *ptsG* strains grew slowly on glucose as sole carbon source.

Because all of the selections for the mutations described above were for tetracycline resistance, it was necessary to render strains tetracycline sensitive between steps if multiple mutations were to be introduced. To accomplish this, tetracycline-resistant strains were plated to TCS medium (15 g/L agar, 5 g/L Bacto tryptone, 5 g/L yeast extract, 50 mg/L chlortetracycline hydrochloride, 10 g/L NaCl, 10 g/L $NaH_2PO_4 \cdot H_2O$, 12 mg/L fusaric acid, and 0.1 mM $ZnCl_2$) which selects for tetracycline sensitive mutants (described in Maloy and Nunn, 1981, *J. Bacteriol.*, 145:1110-1112, which is incorporated herein by reference in its entirety). Colonies arising on this medium were purified by restreaking to the same medium, then checking individual colonies for tetracycline sensitivity by plating to LB media with and without 12.5 µg/mL tetracycline.

The scheme described above for the production of strains containing combinations of the *manXYZ*, *ptsG*, and *Δnag* mutations is presented schematically in Fig. 3.

Example 2

The following Example describes the cloning and overexpression of the *glmS* gene and the integration of the T7-*glmS* gene cassette into the *E. coli* chromosome.

Cloning and Overexpression of the glmS Gene.

Using information obtained from the published sequence of the *glmS* gene (Walker *et al.*, 1984, *Biochem. J.*, 224:799-815, which is incorporated herein by reference in its entirety), primers were synthesized to amplify the gene from genomic DNA isolated from strain W3110 (Table 1) using the polymerase chain reaction (PCR). The primers used for PCR amplification were designated Up1 and Lo8 and had the following sequences:

Up1: 5'-CGGTCTCCCATGTGTGGAATTGTTGGCGC-3' (SEQ ID NO:1)

Lo8: 5'-CTCTAGAGCGTTGATATTCAGTCAATTACAAACA-3' (SEQ ID NO:2)

The Up1 primer contained sequences corresponding to the first twenty nucleotides of the *glmS* gene (represented in nucleotides 10-29 of SEQ ID NO:1) preceded by a *Bsa*I restriction endonuclease recognition site (GGTCTC, represented in nucleotides 2-7 of SEQ ID NO:1). The Lo8 primer contained sequences corresponding to positions between 145 and 171 bases downstream of the *glmS* gene (represented in nucleotides 8-34 of SEQ ID NO:2) preceded by a *Xba*I restriction endonuclease site (TCTAGA, represented in nucleotides 2-7 of SEQ ID NO:2). PCR amplification was conducted using a standard protocol to generate a fragment of DNA containing the *glmS* gene with 171 base pairs of DNA downstream of the gene flanked by *Bsa*I and *Xba*I sites. This DNA fragment was cloned into the vector pCR-Script™SK(+) (Stratagene Cloning Systems, La Jolla, California) using materials and instructions supplied by the manufacturer. The resulting plasmid was designated pKLN23-20.

One consequence of this cloning was that it placed a unique *Sac*I restriction endonuclease site downstream of the gene. This allowed excision of a fragment of DNA containing the *glmS* gene from pKLN23-20 using the restriction endonucleases *Bsa*I and *Sac*I. This fragment was then cloned between the *Nco*I and *Sac*I sites of the expression vector pET-24d(+) (Novagen, Inc., Madison, Wisconsin) to generate plasmid pKLN23-23. The pET-24d(+) expression vector is based on the T7 promoter system (Studier and Moffatt, 1986, *J. Mol. Biol.*, 189:113-130). Cloning in this manner resulted in placement of the *glmS* gene behind the T7-*lac* promoter contained on pET-24d(+). The T7-*lac* promoter is specifically recognized by the T7 RNA polymerase and is only expressed in strains containing

a cloned T7 gene 1, which encodes the T7 RNA polymerase. The cloned T7 polymerase gene is contained on a defective bacteriophage λ phage designated λDE3. Strains in which the λDE3 element is integrated into the chromosome contain the T7 RNA polymerase gene driven by the *lacUV5* promoter. In those strains, expression of the T7 RNA polymerase gene can be induced using the lactose analog isopropylthio-β-D-galactopyranoside (IPTG). Accordingly, addition of IPTG to such cultures results in induction of the T7 RNA polymerase gene and expression of any genes controlled by the T7 or T7-*lac* promoter.

To verify that pKLN23-23 contained the *glmS* gene driven by the T7-*lac* promoter, the plasmid was transferred to strain BL21(DE3) (Novagen, Inc.) (Table 1). Strain BL21(DE3)/pKLN23-23 was grown in duplicate in LB medium containing 50 mg/L kanamycin (kanamycin resistance is encoded by the plasmid). One of the duplicates was induced with 1 mM IPTG; the other was not. When the total proteins were examined from these two cultures by sodium dodecyl sulfate polyacrylamide gel electrophoresis, a prominent protein of approximately 70,000 molecular weight, corresponding to the predicted size for the *glmS* gene product, was observed in cells from the induced culture but not in cells from the uninduced culture. A preliminary enzyme assay from an induced culture indicated several hundred fold higher glucosamine-6-phosphate synthase activity in the induced culture than in what had typically been observed in a wild type strain.

Integration of the T7-glmS Gene Cassette into the E. coli Chromosome.

The *glmS* gene driven by the T7-*lac* (the T7-*glmS* gene cassette) promoter was transferred to the chromosome of *E.*

coli strains by a multistep process. First, the cassette was cloned into plasmid pBRINT-Cm (Balbás *et al.*, 1996, *Gene* 96:65-69), which is incorporated herein by reference in its entirety). The gene cassette was then integrated into the chromosome of strain ATCC47002 (Table 1) by the techniques described by Balbás *et al.*, 1996, *supra*, to generate strains T-71 and T-81 (Table 1). Finally, the gene cassette was transferred to strains of interest by transduction with $P1_{vir}$, as described below.

The T7-*glmS* cassette was excised from pKLN23-23 by performing a partial digest of the plasmid with restriction endonuclease *Bgl*II and a complete digest with restriction endonuclease *Hin*DIII. Plasmid pKLN23-23 contains a *Bgl*II site approximately 20 base pairs upstream of the T7 promoter. The *glmS* gene also contains two *Bgl*II sites. A partial digest with this enzyme was necessary to cut the plasmid upstream of the T7 promoter while avoiding the two internal sites. Plasmid pKLN23-23 also contains a unique *Hin*DIII site downstream of the *glmS* gene. The excised T7-*glmS* cassette was then cloned between the unique *Bam*HI and *Hin*DIII sites of pBRINT-Cm. This resulted in the production of plasmids designated pKLN23-27 and pKLN23-28. Plasmids pKLN23-27 and pKLN23-28 contain the T7-*glmS* cassette in addition to a chloramphenicol resistance determinant flanked by the 5'- and 3'-termini of the *E. coli lacZ* gene.

Strain ATCC 47002 (Table 1) contains mutations that confer upon it an inability to maintain plasmids such as pBRINT-Cm which contain a ColE1 origin of replication. When such plasmids are transferred to this strain, selection for genetic markers contained on the plasmid results in integration of the plasmid into the chromosome (Balbás *et al.*,

1996, *supra*). As mentioned above, plasmids pKLN23-27 and -28 contain the T7-*glmS* cassette and a chloramphenicol resistance determinant flanked by the 5'- and 3'-termini of the *E. coli* *lacZ* gene. The *lacZ* sequences target the incoming DNA to the *lacZ* gene contained in the chromosome. Integration at the *lacZ* locus replaces the intact *lacZ* gene, which encodes the enzyme β-galactosidase, with a partial *lacZ* gene interrupted by the T7-*glmS*-Cm cassette. As a result, integration at *lacZ* results in the strain becoming β-galactosidase negative. The plasmid also contains an ampicillin resistance determinant remote from the 5'-*lacZ*-T7-*glmS*-Cm-*lacZ*-3' cassette. Integration at *lacZ* and plasmid loss also results in ampicillin sensitivity.

Plasmids pKLN23-27 and -28 were transferred to strain ATCC 47002, and cells were plated to LB medium containing 10 µg/mL chloramphenicol, 1 mM IPTG, and 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The X-gal contained in the medium is a chromogenic β-galactosidase substrate. Hydrolysis of X-gal by β-galactosidase results in a blue derivative. Inclusion of X-gal and IPTG, which induces the native *lacZ* gene, results in blue *lacZ*-positive colonies and white *lacZ*-negative colonies. White (*lacZ*-negative) chloramphenicol resistant colonies were then selected and purified. The strains were then verified for sensitivity to ampicillin by plating to LB media with and without 100 µg/mL ampicillin. DNA integration was further confirmed using a PCR scheme as described by Balbás *et al.*, 1996, *supra*. Integrants T-71 and T-81 (Table 1) resulted from the integration of the desired segments of plasmids pKLN23-27 and pKLN23-28, respectively, into the chromosome of ATCC 47002.

The T7-*glmS*-Cm cassette was then transferred to strains W3110(DE3), 7101-9(DE3), 7101-17(DE3), and 2123-4(DE3) by $P1_{vir}$ transduction, as described in Example 1, using lysates prepared on strains T-71 and T-81. These strains contain various combinations of the Δ*nag*, *manXYZ*, and *ptsG* mutations in addition to the λDE3 element necessary for expression from the T7-*lac* promoter. The λDE3 element was introduced to these strains using the λDE3 lysogenization kit produced by Novagen, Inc. (Madison, Wisconsin). Transductants were selected on LB agar plates containing 30 μg/mL chloramphenicol and 10 mM sodium citrate. Loss of β-galactosidase activity was verified on plates containing X-gal and IPTG and DNA integration was further confirmed using a PCR scheme as described by Balbás *et al.*, 1996, *supra*.

Glucosamine-6-phosphate synthase activity was measured in production strains containing integrated T7-*glmS* cassettes after growth in LB medium with and without IPTG (Table 2). Glucosamine-6-phosphate synthase was assayed in crude cell extracts using either colorimetric or spectrophotometric assays (Badet *et al.*, 1987, *Biochemistry* 26:1940-1948) as described below. The extracts used for those assays were prepared by suspending washed cell pellets in 5 mL of 0.1 M KH_2PO_4/K_2HPO_4, pH 7.5 per gram of wet cell paste, passing the suspension through a French press at 16,000 psi, and centrifuging the disrupted cell suspension at 35,000-40,000 x *g* for 15 to 20 minutes. The supernatant was used as the source of enzyme for the assay.

For a colorimetric assay, 1 mL reactions were prepared containing 45 mM KH_2PO_4/K_2HPO_4, 20 mM fructose-6-phosphate, 15 mM L-glutamine, 2.5 mM EDTA, pH 7.5, and cell extract. The reactions were incubated at 37°C for 20 minutes and stopped by

boiling for 4 minutes. The resulting precipitate was removed by centrifugation and the supernatant was assayed for glucosamine-6-phosphate by a modification of the method of Elson and Morgan (1933, *Biochem. J.* 27:1824-1828) essentially as described by Zalkin (1985, *Meth. Enzymol.* 113:278-281), both publications of which are incorporated herein by reference in their entireties. To 100 µL of the above supernatant was added 12.5 µL of saturated $NaHCO_3$ and 12.5 µL of cold, freshly prepared 5% aqueous acetic anhydride. After incubating for 3 minutes at room temperature, the mixture was boiled for 3 minutes to drive off excess acetic anhydride. After cooling to room temperature, 150 µL of 0.8 M potassium borate, pH 9.2 (0.8 M H_3BO_3 adjusted to pH 9.2 with KOH) was added and the mixture was boiled for 3 minutes. After cooling to room temperature, 1.25 mL Ehrlich's reagent (1% *p*-dimethylaminobenzaldehyde in glacial acetic acid containing 0.125 N HCl) was added to each tube. After incubating at 37°C for 30 minutes, the absorbance at 585 nm was measured and the amount of glucosamine-6-phosphate formed was determined using a standard curve. In the absence of the substrate, fructose-6-phosphate, or when boiled extract was used in the assay, no significant absorbance at 585 nm was observed.

In the spectrophotometric assay, 1 mL reactions containing 50 mM KH_2PO_4/K_2HPO_4, 10 mM fructose-6-phosphate, 6 mM L-glutamine, 10 mM KCl, 0.6 mM acetylpyridine adenine dinucleotide (APAD), and 50-60 Units of L-glutamic dehydrogenase (Sigma Type II from bovine liver) were run at room temperature. The activity was followed by monitoring the absorbance at 365 nm after the addition of extract and corrected for the small absorbance increase observed in the

absence of extract. The activity was calculated using a molar extinction coefficient for APAD of 9100.

Table 2

Glucosamine 6-Phosphate Synthase Activity in Production Strains Containing Integrated T7-*glmS* Cassettes

Strain	Host Genotype	Activity, (µmole per minute per mL of extract)	
		- IPTG	+ IPTG
2123-5	DE3	23	64
2123-6	DE3	4	4
2123-7	DE3	23	96
2123-8	DE3	25	89
2123-9	DE3 *Δnag*	26	58
2123-10	DE3 *Δnag*	33	67
2123-11	DE3 *Δnag manXYZ*	32	59
2123-12	DE3 *Δnag manXYZ*	17	67
2123-13	DE3 *Δnag manXYZ ptsG*	21	68
2123-14	DE3 *Δnag manXYZ ptsG*	20	88

Table 2 shows that, on average, the activity of glucosamine-6-phosphate synthase in production strains containing integrated T7-*glmS* cassettes was about three- to four-fold higher with IPTG induction than without. The activities were substantially higher than those obtained with a wild type *glmS* strain driven by its native promoter, which typically were in the range of 0.05-0.1 µmole per minute per mL of extract. One of the strains, 2123-6, apparently suffered an aberrant integration event since the activity was lower than that observed in the other strains and was not influenced by the presence of IPTG in the medium.

Example 3

The following example shows the effect of strain genotype on glucosamine accumulation.

Strains with T7-*glmS* integrants, produced as described in Example 2, as well as the corresponding parent strains without integrated DNA, were grown in shake flasks containing M9A medium (14 g/L K_2HPO_4, 16 g/L KH_2PO_4, 1 g/L $Na_3Citrate{\cdot}2H_2O$, 5 g/L $(NH_4)_2SO_4$, pH 7.0) supplemented with 20 g/L glucose, 10 mM $MgSO_4$, 1 mM $CaCl_2$, and 1 mM IPTG. Samples were taken periodically over the course of two days, and the glucosamine concentration in the culture supernatant was measured using the modified Elson-Morgan assay as described in Example 2. Samples were assayed with and without acetic anhydride treatment, and the amount of glucosamine present was determined from the net absorbance using a standard curve.

Glucosamine concentrations after 24 hours of cultivation, at which time the concentration peaked, are indicated in Table 3. The results shown in Table 3 indicate that for significant glucosamine production to occur, the T7-*glmS* gene cassette must be present. The data also indicate that the presence of the Δ*nag* mutation in the host results in a significant increase in glucosamine accumulation compared with its absence. Little effect of the *manXYZ* mutation was observed in this experiment. In further shake flask experiments, however, strain 2123-12 accumulated slightly higher glucosamine concentrations on a consistent basis.

Table 3

Glucosamine in Culture Supernatants of Production Strains

Strain	Genotype	Glucosamine Concentration, mg/L (24 hours)
2123-5	DE3, T-71 integrant	21
2123-7	DE3, T-71 integrant	23
2123-9	DE3 Δ*nag*, T-71 integrant	67
2123-10	DE3 Δ*nag*, T-81 integrant	80
2123-11	DE3 Δ*nag manXYZ*, T-71 integrant	65
2123-12	DE3 Δ*nag manXYZ*, T-81 integrant	63
W3110(DE3)	DE3, no integrant	4
7101-9(DE3)	DE3 Δ*nag*, no integrant	0
7101-17(DE3)	DE3 Δ*nag manXYZ*, no integrant	0

Example 4

The following example demonstrates the effect feeding nutrients to the cultures has on glucosamine accumulation.

In early experiments, it was observed that glucosamine accumulation ceased when glucose was depleted from cultures. In the experiment summarized by Table 4 and Fig. 4, it was found that increased glucosamine accumulation could be accomplished by feeding additional glucose and ammonium sulfate as they became depleted. For this experiment, strain 2123-12 was grown in M9A medium supplemented with 10 mM $MgSO_4$, 1 mM $CaCl_2$, and 1 mM IPTG. Initial glucose concentrations and feeding regimens were varied as indicated in Table 4. In one of the flasks, the initial ammonium sulfate concentration was 10 g/L rather than the 5 g/L normally used in M9A medium. Glucose concentration was monitored in shake flasks during cultivation using Diastix® glucose test strips (Bayer Corporation Diagnostics Division, Elkhart, Indiana). When the glucose concentration was at or near depletion (<5 g/L remaining), glucose and/or ammonium sulfate were supplemented as indicated in Table 4. pH was also monitored during cultivation. When the pH varied significantly from the

initial pH of 7.0, it was adjusted to 7.0 with sodium hydroxide.

Table 4

Shake Flask Experiment to Examine the Effect of Glucose Feeding

Flask No.	Initial Glucose, g/L	Initial Ammonium Sulfate, g/L	Feed
1	20	5	None
2	50	5	None
3	50	10	None
4	20	5	20g/L Glucose
5	20	5	20 g/L Glucose + 5 g/L $AmSO_4$

As Fig. 4 indicates, increasing the supply of glucose had a positive effect on glucosamine accumulation. By periodically feeding with glucose and ammonium sulfate (20 g/L and 5 g/L additions, respectively), a maximum accumulation of 0.4 g/L of glucosamine was observed, approximately four-fold higher than what was observed in the absence of feeding.

Example 5

The following example describes the isolation of mutant *glmS* genes encoding glucosamine-6-phosphate synthase enzymes with decreased sensitivity to glucosamine-6-phosphate product inhibition.

White (1968, *Biochem. J.*, 106:847-858) first demonstrated that glucosamine-6-phosphate synthase was inhibited by glucosamine-6-phosphate. Using the spectrophotometric assay for glucosamine-6-phosphate synthase as described in Example 2, the effects of glucosamine-6-phosphate and glucosamine on glucosamine-6-phosphate synthase were measured. For determination of product inhibition, assays were run in the presence of various concentrations of added glucosamine-6-phosphate.

As indicated in Fig. 5, the enzyme is significantly inhibited by glucosamine-6-phosphate and slightly inhibited by glucosamine. These results are similar to those obtained by White, 1968, *supra*. This inhibition may be a key factor in limiting glucosamine accumulation in the glucosamine production strains.

To further increase glucosamine synthesis in production strains, efforts were made to isolate mutants of the *glmS* gene encoding glucosamine-6-phosphate synthase variants with reduced product inhibition. To accomplish this, the cloned gene was amplified using the technique of error-prone PCR. In this method, the gene is amplified under conditions that lead to a high frequency of misincorporation errors by the DNA polymerase used for the amplification. As a result, a high frequency of mutations are obtained in the PCR products.

Plasmid pKLN23-28 contains a unique *Spe*I restriction endonuclease site 25 base pairs upstream of the T7 promoter and 111 base pairs upstream of the start of the *glmS* gene. The plasmid also contains a unique *Hin*DIII site 177 base pairs downstream of the *glmS* gene. PCR primers of the following sequences were synthesized to correspond to regions just upstream of the *Spe*I and downstream of the *Hin*DIII sites, respectively:

5'-ATGGATGAGCAGACGATGGT-3' (SEQ ID NO:3)

5'-CCTCGAGGTCGACGGTATC-3' (SEQ ID NO:4)

Amplification with these primers (SEQ ID NO:3 and SEQ ID NO:4) allowed mutagenesis of a 2247 base pair region that included the entire *glmS* gene. PCR conditions were as described by Moore and Arnold, 1996, *Nature Biotechnology* 14:458-467, which is incorporated herein by reference in its entirety. Briefly, a 100 µL solution was prepared containing

1 mM each of the four deoxynucleotide triphosphates, 16.6 mM ammonium sulfate, 67 mM Tris-HCl, pH 8.8, 6.1 mM $MgCl_2$, 6.7 µM EDTA, 10 mM β-mercaptoethanol, 10 µL DMSO, 30 ng each of the primers (SEQ ID NO:3 and SEQ ID NO:4), either 7 or 35 ng of plasmid pKLN23-28 linearized with *Kpn* I, and 2.5 Units of *Taq* DNA polymerase (Perkin Elmer-Cetus, Emeryville, California). The reaction mixture was covered with 70µL of mineral oil and placed in a thermocycler, where the following steps were repeated for 25 cycles:

1 minute at 94°C
1 minute at 42°C
2 minutes at 72°C

Under these conditions, an error frequency of approximately one mutation per 1000 base pairs has been reported (Moore and Arnold, 1996, *supra*). The product of the reaction was recovered, purified, and digested with *Spe*I and *Hin*DIII, and cloned into the *Spe*I-*Hin*DIII backbone fragment of pKLN23-28, which effectively substitutes for the wild type *glmS* gene on the *Spe*I-*Hin*DIII fragment of pKLN23-28. The cloned DNA was used to transform strain NovaBlue (Novagen, Inc., Madison, Wisconsin), and the transformed cells were plated to LB agar containing ampicillin. A total of 9000 plasmid-containing colonies were pooled from the ampicillin plates and plasmid DNA was prepared from the pooled cells to generate a library of pKLN23-28 derivative plasmids containing mutations in the cloned *glmS* gene.

The mutant plasmids generated by error-prone PCR were screened for their ability to confer increased glucosamine production in a Δ*nag manXYZ* DE3 host background. This screen was in the form of a bioassay in which the ability of plasmid-

containing strains to crossfeed glucosamine-requiring strains of *E. coli* was assessed.

Strains of *E. coli* (Sarvas, 1971, *J. Bacteriol.* 105:467-471; Wu and Wu, 1971, *J. Bacteriol.* 105:455-466) and *Bacillus subtilis* (Freese *et al.*, 1970, *J. Bacteriol.* 101:1046-1062) which are defective for glucosamine-6-phosphate synthase require glucosamine or N-acetylglucosamine for growth. A glucosamine-requiring strain of *E. coli* was isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG). Strain LE392 (Table 1) was grown in LB medium to a cell density of 6 x 10^8 cells per mL. 50 µL of 2.5 mg/mL NG dissolved in methanol was added to 2 mL of this culture and the mixture was incubated at 37°C for 20 minutes. This treatment resulted in about 10% survival of the strain. The mutagenized cells were harvested by centrifugation, and the cells were washed twice by suspension in 0.9% NaCl and recentrifugation. The washed cells were diluted and plated to nutrient agar medium (NA; 5 g/L Bacto peptone, 3 g/L beef extract, 15 g/L agar) containing 0.2 g/L N-acetylglucosamine at a density of between 50 and 200 colony forming units per plate. Approximately 13,000 colonies were plated. These colonies were replica-plated to NA agar with and without 0.2 g/L N-acetylglucosamine. Twenty-two colonies grew on NA with 0.2 g/L N-acetylglucosamine but not on NA without 0.2 g/L N-acetylglucosamine. These colonies were purified by streaking to NA with 0.2 g/L N-acetylglucosamine, and their growth phenotype was rechecked. Of the original 22 colonies selected, five had the phenotype expected of a *glmS* mutant of LE392. They failed to grow on NA but grew on NA supplemented with 0.2 g/L of glucosamine or 0.2 g/L N-acetylglucosamine. They also failed to grow on glucose minimal agar, but grew on

glucose minimal agar supplemented with 0.2 g/L N-acetylglucosamine. One of these mutants was designated 2123-16 (Table 1).

For the cross-feeding assay, agar plates containing either glycerol or fructose as the principle carbon source for growth were overlaid with cells from a culture of strain 2123-16, the glucosamine-requiring strain isolated as described above. Glucosamine-producing strains were stabbed into the agar and the ability to produce glucosamine was assessed based on the size of the "halo" of growth of the indicator strain surrounding the stab. Those stabs surrounded by larger halos were considered to produce greater amounts of glucosamine.

The media used for the cross-feeding assays consisted of M9 minimal medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 40 mg/L of L-methionine (required for growth of strains LE392 or 2123-16) and 2 g/L of either glycerol or fructose. These plates were overlaid with strain 2123-16 as follows. A culture of strain 2123-16 was grown overnight at 37°C in LB medium containing 1 g/L N-acetylglucosamine. The culture was harvested by centrifugation, and the cells were washed twice by suspension in 0.9% NaCl and recentrifugation. The washed cells were suspended in the original volume of 0.9% NaCl. For each plate to be overlaid, 0.1 mL of washed cell suspension was mixed with 3 mL of molten (50°C) F-top agar (8 g/L NaCl, 8 g/L agar) and poured onto the plate.

The library of pKLN23-28 mutant plasmids was transferred to strain 7101-17(DE3) and transformed cells were plated to LB agar containing 100 µg/mL ampicillin. Each colony arising on these plates contained an individual member of the mutant plasmid library. The colonies were screened by picking them

from the LB + ampicillin plates and stabbing them sequentially into:

(1) LB agar + ampicillin;

(2) glycerol minimal agar overlaid with strain 2123-16; and,

(3) fructose minimal agar overlaid with strain 2123-16

All plates were incubated for about 24 hours at 37°C. After this incubation period, halos of growth of the 2123-16 indicator strain could be observed surrounding the stabs in the overlaid plates. Those colonies giving rise to the larger halos were picked from the corresponding LB + ampicillin plate and streaked for purification. In an initial screen, 4368 mutant candidates were screened, and 96 initial candidates were identified. Upon rescreening those, 30 appeared to be superior to the rest, *i.e.* resulted in larger halos of the indicator strain.

Enzyme assays performed with six of the plasmid-containing strains isolated as described above indicated that three of the strains were less sensitive to inhibition by glucosamine-6-phosphate than the enzyme from the control strain 7101-17(DE3)/pKLN23-28. The strains were grown overnight in LB broth containing 100 µg/mL ampicillin and 1 mM IPTG. Extracts prepared from cells harvested from those cultures were assayed for glucosamine-6-phosphate synthase using the spectrophotometric assay (described in Example 2) in the presence and absence of added glucosamine-6-phosphate. The mutant clones designated 11C, 65A, and 8A were significantly less sensitive to glucosamine-6-phosphate than the control strain (Fig. 6). Other mutants were not distinguishable from the control by this assay.

Example 6

The following example describes the construction and characterization of glucosamine production strains with mutations in *glmS* which result in reduced product inhibition.

Plasmid DNA isolated from clones 11C, 52B, and 8A described above were transferred to strain ATCC 47002, which had been used previously to integrate the cloned T7-*glmS* construct into the *E. coli* chromosome. Integration was readily accomplished using the methods described in Example 2, and the integrated DNA was transferred to strain 7101-17(DE3) by P1 transduction as described in Example 1. These procedures produced strains that have the same genotype as strain 2123-12 except for the presence of mutations in the *glmS* gene generated by PCR. These new mutant production strains were designated 2123-49, 2323-51, and 2123-54, respectively. A summary of the strain construction strategy is presented in Fig. 7.

Strains 2123-12, 2123-49, 2123-51, and 2123-54 were grown overnight in LB broth containing 1 mM IPTG. Extracts prepared from cells harvested from those cultures were assayed for glucosamine-6-phosphate synthase using the spectrophotometric assay described in Example 2 in the presence and absence of added glucosamine-6-phosphate. The results of these assays are shown in Fig. 8.

Glucosamine production in these mutants was significantly elevated compared to that in 2123-12. When glucosamine production was assayed in shake flask cultures grown using the glucose and ammonium sulfate feeding protocol previously described in Example 4, when the cultures were grown to a cell density (measured as $O.D._{600}$) of about 14 (about 8.4 g/L by dry cell weight), strains 2123-49, 2123-51, and 2123-54 produced

1.5, 2.4, and 5.8 g/L glucosamine, respectively (Fig. 9) compared with 0.3 g/L for 2123-12.

Example 7

The following example describes the production of yet another strain with a mutation in *glmS* which results in reduced product inhibition.

An additional 6,344 colonies containing mutant plasmids generated by error-prone PCR as described in Example 5 were screened using the cross-feeding assay, also described in Example 5. Fifty four colonies resulted in larger halos than the rest of the colonies. DNA was isolated from all 54 colonies and strains isogenic to 2123-12 except for the mutations in *glmS* were constructed as described in Example 6.

Glucosamine production in most of these mutants was significantly elevated compared to strain 2123-12. Among the newly isolated mutants, the strain that produced the most glucosamine was a strain designated 2123-124. This strain produced 3.6 g/L of glucosamine when production was assayed in shake flasks using the glucose and ammonium sulfate feeding protocol described in Example 4 compared with 4.3 g/L for strain 2123-54 in a side-by-side experiment.

Example 8

The following example describes the sequencing of the cloned wild type *glmS* gene present in plasmid pKLN23-28. In addition, the sequences present in plasmids pKLN23-49, pKLN23-54, and pKLN23-124, containing the mutant *glmS* genes used to construct strains 2123-49, 2123-54, and 2123-124, respectively were sequenced and are described.

DNA sequencing reactions were performed using the Applied Biosystems (ABI) Prism Dye Terminator Cycle sequencing method with AmpliTaq DNA polymerase. The extended products were separated by gel electrophoresis on an ABI DNA sequencer 373A or 377. Sequences were analyzed using ABI Sequencing Analysis 3.0 software from ABI and Sequencher 3.1 from Gene Codes.

The primers used for sequencing were as follows:

PK-1: 5′-TGGATGAGCAGACGATGG-3′ (SEQ ID NO:5)

PK-2: 5′-TCCGTCACAGGTATTTATTC-3′ (SEQ ID NO:6)

PK-3: 5′-AGCTGCGTGGTGCGTAC-3′ (SEQ ID NO:7)

PK-4: 5′-GGACCGTGTTTCAGTTCG-3′ (SEQ ID NO:8)

PK-5A: 5′-GCCGTGGCGATCAGTAC-3′ (SEQ ID NO:9)

PK-6A: 5′-GCCAATCACCAGCGGAC-3′ (SEQ ID NO:10)

PK-7: 5′-ATGGTTTCCCGCTACTGG-3′ (SEQ ID NO:11)

PK-8: 5′-GAACCAAGGTAACCCAGC-3′ (SEQ ID NO:12)

The nucleotide sequence of plasmid pKLN23-28, containing the wild-type *glmS* gene, was determined to be a 7408 bp nucleic acid sequence represented herein as SEQ ID NO:13. The 2184 base pairs between positions 1130 and 3313 of SEQ ID NO:13 were determined using the primers described above. The nucleic acid molecule representing positions 1130-3313 of SEQ ID NO:13 is referred to herein as nglmS-28_{2184} and is further identified as SEQ ID NO:14. nglmS-28_{2184} was shown to include the *Spe*I and *Hin*DIII sites used to construct the mutant plasmids as described in Example 5. The remaining DNA sequence of SEQ ID NO:13 is based on the known sequences of the vectors used for the construction of pKLN23-28. The same 2184 base pair region was sequenced in plasmids pKLN23-49, pKLN23-54, and pKLN23-124. It is noted that for the discussion of the mutant *glmS* genes of these plasmids (Table 5), the specific nucleotide position of mutations in the

nucleotide sequence of the mutant *glmS*-containing plasmids will be described using SEQ ID NO:13 as a reference.

SEQ ID NOs:13 and 14 contain an open reading frame that encodes the *glmS* gene product (i.e., GlcN6P synthase enzyme) which is a nucleic acid molecule referred to herein as nglmS-28_{1830}, the nucleic acid sequence of which is represented by SEQ ID NO:15. SEQ ID NO:15 spans nucleotides 1253 to 3082 of SEQ ID NO:13, with an initiation codon spanning from nucleotides 1253-1255 and a termination codon spanning from nucleotides 3080-3082. SEQ ID NO:15 encodes a protein of 609 amino acids referred to herein as GlcN6P-S-28, the deduced amino acid sequence of which is represented herein as SEQ ID NO:16. It is noted that for the discussion of the mutant *glmS* gene products produced by the mutant strains described herein, specific mutations in the amino acid sequence of the mutant *glmS* gene products will be described using SEQ ID NO:16 as a reference.

The primers described above correspond to the following nucleotide positions of SEQ ID NO:13:

PK-1 (SEQ ID NO:5): positions 1087-1104 of SEQ ID NO:13;

PK-2 (SEQ ID NO:6): positions 3378-3359 of a nucleic acid sequence complementary to SEQ ID NO:13;

PK-3 (SEQ ID NO:7): positions 1707-1723 of SEQ ID NO:13;

PK-4 (SEQ ID NO:8): positions 2772-2755 of a nucleic acid sequence complementary to SEQ ID NO:13;

PK-5A (SEQ ID NO:9): positions 2667-2683 of SEQ ID NO:13;

PK-6A (SEQ ID NO:10): positions 1798-1782 of a nucleic acid sequence complementary to SEQ ID NO:13;

PK-7 (SEQ ID NO:11): positions 2177-2194 of SEQ ID NO:13;

PK-8 (SEQ ID NO:12): positions 2364-2347 of a nucleic acid sequence complementary to SEQ ID NO:13.

The nucleic acid sequence of nucleic acid molecule nglmS-28_{1830} (SEQ ID NO:15, or positions 1253-3082 of SEQ ID NO:13) from pKLN23-28, differs from the published sequence (Walker, J.E, et al., 1984, "DNA sequence around the *Escherichia coli unc* operon", *Biochem. J.* 224:799-815) at positions 2509 and 2510 (with reference to SEQ ID NO:13). The nucleotides for pKLN23-28 at these positions as determined in this example were G and C, respectively, while those reported in the published sequence were C and G. Otherwise, the published and determined sequences of the *glmS* gene were identical. The sequences determined upstream and downstream from the *glmS* gene were those expected based on the known sequences of the vectors used for the construction of pKLN23-28 and the methods used to construct the plasmid.

The nucleotide sequences for the mutant *glmS* genes for plasmids pKLN23-49, pKLN23-54, and pKLN23-124 were determined as described above for pKLN23-28. Mutations were found in each of those plasmids. The mutations and the predicted amino acid changes in the corresponding mutant *glmS* gene products, as compared to the wild-type sequence determined for pKLN23-28 (SEQ ID NO:13) are summarized in Table 5.

Table 5

Mutations in *glmS* Genes of Glucosamine-Overproducing strains.

Plasmid	Position*	Base Change	Amino Acid Change (Position**)
pKLN23-49	1263	T to C	Ile to Thr (4)
	2067	T to C	Ile to Thr (272)
	2600	T to C	Ser to Pro (450)
pKLN23-54	1367	G to A	Ala to Thr (39)
	2000	C to T	Arg to Cys (250)
	2239	T to C	Silent (329)
	2666	G to A	Gly to Ser (472)
	3264	A to G	Outside gene
pKLN23-124	1525	T to C	Silent (91)
	2658	T to C	Leu to Pro (469)
	3280	G to A	Outside gene

*Refers to nucleic acid position as indicated in the sequence of pKLN23-28 (SEQ ID NO:13)
**The *glmS* gene (nglmS-28_{1830}; SEQ ID NO:15) encodes a protein of 609 amino acids in length (SEQ ID NO:16); the methionine residue at position 1 is removed by a hydrolase.

Plasmid pKLN23-49 contains a 2184 bp nucleic acid molecule referred to herein as nglmS-49_{2184}, which comprises a mutant *glmS* gene. The nucleic acid sequence of nglmS-49_{2184} is represented herein as SEQ ID NO:17. A nucleic acid molecule spanning from nucleotide 124 through 1953 of SEQ ID NO:17, referred to herein as nglmS-49_{1830}, represents an open reading frame encoding a mutant glucosamine-6-phosphate synthase of the present invention, with an initiation codon spanning from nucleotides 124-126 and a termination codon spanning from nucleotides 1951-1953 of SEQ ID NO:17. The nucleic acid sequence of nglmS-49_{1830} is represented herein as SEQ ID NO:18. SEQ ID NO:18 encodes a mutant glucosamine-6-phosphate synthase protein of 609 amino acids referred to herein as GlcN6P-S-49,

the deduced amino acid sequence of which is represented herein as SEQ ID NO:19. SEQ ID NO:17 has a nucleic acid sequence that is identical to positions 1130 through 3313 of SEQ ID NO:13 (i.e., SEQ ID NO:14), except for the mutations as indicated for plasmid pKLN23-49 in Table 5. SEQ ID NO:18 has a nucleic acid sequence that is identical to positions 1253 through 3082 of SEQ ID NO:13 (i.e., SEQ ID NO:15), except for the mutations as indicated for plasmid pKLN23-49 in Table 5.

Plasmid pKLN23-54 contains a 2184 bp nucleic acid molecule referred to herein as nglmS-54_{2184}, which comprises a mutant *glmS* gene. The nucleic acid sequence of nglmS-54_{2184} is represented herein as SEQ ID NO:20. A nucleic acid molecule spanning from nucleotide 124 through 1953 of SEQ ID NO:20, referred to herein as nglmS-54_{1830}, represents an open reading frame encoding a mutant glucosamine-6-phosphate synthase of the present invention, with an initiation codon spanning from nucleotides 124-126 and a termination codon spanning from nucleotides 1951-1953 of SEQ ID NO:20. The nucleic acid sequence of nglmS-54_{1830} is represented herein as SEQ ID NO:21. SEQ ID NO:21 encodes a mutant glucosamine-6-phosphate synthase protein of 609 amino acids referred to herein as GlcN6P-S-54, the deduced amino acid sequence of which is represented herein as SEQ ID NO:22. SEQ ID NO:20 has a nucleic acid sequence that is identical to positions 1130 through 3313 of SEQ ID NO:13 (i.e., SEQ ID NO:14), except for the mutations as indicated for plasmid pKLN23-54 in Table 5. SEQ ID NO:21 has a nucleic acid sequence that is identical to positions 1253 through 3082 of SEQ ID NO:13 (i.e., SEQ ID NO:15), except for the mutations as indicated for plasmid pKLN23-54 in Table 5.

Plasmid pKLN23-124 contains a 2184 bp nucleic acid molecule referred to herein as nglmS-124_{2184}, which comprises

a mutant *glmS* gene. The nucleic acid sequence of nglmS-124_{2184} is represented herein as SEQ ID NO:23. A nucleic acid molecule spanning from nucleotide 124 through 1953 of SEQ ID NO:23, referred to herein as nglmS-124_{1830}, represents an open reading frame encoding a mutant glucosamine-6-phosphate synthase of the present invention, with an initiation codon spanning from nucleotides 124-126 and a termination codon spanning from nucleotides 1951-1953 of SEQ ID NO:23. The nucleic acid sequence of nglmS-124_{1830} is represented herein as SEQ ID NO:24. SEQ ID NO:24 encodes a mutant glucosamine-6-phosphate synthase protein of 609 amino acids referred to herein as GlcN6P-S-124, the deduced amino acid sequence of which is represented herein as SEQ ID NO:25. SEQ ID NO:23 has a nucleic acid sequence that is identical to positions 1130 through 3313 of SEQ ID NO:13 (i.e., SEQ ID NO:14), except for the mutations as indicated for plasmid pKLN23-124 in Table 5. SEQ ID NO:24 has a nucleic acid sequence that is identical to positions 1253 through 3082 of SEQ ID NO:13 (i.e., SEQ ID NO:15), except for the mutations as indicated for plasmid pKLN23-124 in Table 5.

To verify that the same mutations were present in the strains into which the mutant *glmS* genes were integrated into the chromosome, PCR products were generated from genomic DNA isolated from strains 2123-49, 2123-54, and 2123-124. For PCR amplification, the primers listed in Example 3 for the mutagenesis of the gene (SEQ ID NO:3 and SEQ ID NO:4) were used. PCR reactions were carried out in 50 µL reactions consisting of 20 mM Tris·HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/mL nuclease-free bovine serum albumin, 0.05 mM each deoxynucleotide triphosphate, 2 µM each primer, 1.25 U cloned

Pfu DNA polymerase (Stratagene), and 160 ng of genomic DNA. The complete reactions were placed in a RoboCycler Gradient 96 Temperature Cycler (Stratagene). After 3 minutes at 94°C, the following three steps were repeated for 30 cycles: (1) 30 seconds at 94°C; (2) 30 seconds at 47°C; and (3) 2 minutes at 72°. This was followed with a 7 minute incubation at 72°C.

The resulting DNA contained the expected amplification product in addition to extraneous products. The product containing the *glmS* gene was purified using a QIAquick PCR purification kit, followed by electrophoresis of the purified product on an agarose gel, isolation of the correct band using a QIAquick gel extraction kit, and reamplification using this isolated DNA as a template. The reactions with the isolated DNA were amplified in a similar fashion as the original amplification described above, except that 40 ng of DNA was used as template, and only 20 cycles of amplification were performed. The product from this second amplification reaction was recovered as described above.

The presence of mutations in the genomic DNA was verified using primers specific for the DNA regions containing the mutations identified in the plasmids. For 2123-49, these included primers PK-1 (SEQ ID NO:5), PK-3 (SEQ ID NO:7), PK-4 (SEQ ID NO:8), and PK-5A (SEQ ID NO:9). For 2123-124, primers PK-1 (SEQ ID NO:5), PK-4 (SEQ ID NO:8) and PK-5 (SEQ ID NO:9) were used. For 2123-54, the entire PCR product was sequenced using all eight primers described earlier (SEQ ID NOs:5-12). Sequencing of the PCR products confirmed the presence of the mutations identified from the plasmids and listed in Table 5.

Example 9

This example describes the construction of strains containing a mutant *glmS* gene encoding a product containing only the glycine to serine alteration at position 472 (SEQ ID NO:22) from strain 2123-54.

As indicated in Table 5, the only amino acid change in the GlcN6P synthase enzyme for strain 2123-124 (GlcN6P-S-124) is a leucine to proline alteration at position 469 (SEQ ID NO:25), unambiguously defining this mutation as being responsible for the overproduction of glucosamine by strain 2123-124. This would suggest the possibility that the glycine to serine alteration at position 472 (gly→ser472; SEQ ID NO:22) of GlcN6P-S-54 in strain 2123-54 was likewise responsible for the glucosamine overproduction phenotype for this strain. In an effort to demonstrate this, the alteration was isolated away from the other two amino acid alterations in the GlcN6P-S-54 amino acid sequence (SEQ ID NO:22) of strain 2123-54 (i.e., Ala→Thr39 and Arg→Cys250) by digesting plasmid pKLN23-54 with *Eco*RI and *Hin*DIII. These enzymes each have unique cleavage sites on the plasmid and cut at positions 2241 and 3305, respectively (positions indicated with respect to the equivalent positions in SEQ ID NO:13 for pKLN23-28), resulting in fragments of 1064 and 6344 base pairs. The smaller fragment contains mutations in which the gly→ser472 alteration is the only amino acid change in this portion of GlcN6P-S-54. This smaller fragment was ligated to the corresponding larger fragment from pKLN23-28 containing the wild type *glmS* gene.

Two plasmids resulting from this ligation were designated pKLN23-149 and pKLN23-151. Sequencing the DNA from these plasmids using primers PK-1 (SEQ ID NO:5), PK-3 (SEQ ID NO:7),

and PK-4 (SEQ ID NO:8) verified that these plasmids contained the mutation at position 2666 present in plasmid pKLN23-54 but not the mutations at positions 1367 and 2000 (Table 5 with reference to SEQ ID NO:13).

The nucleic acid sequence of the 2184 base pairs between positions 1130 and 3313 of plasmid pKLN23-149 (these positions being determined relative to the equivalent positions in SEQ ID NO:13) are referred to herein as nucleic acid molecule nglmS-149_{2184}, the nucleic acid sequence of which is represented by SEQ ID NO:26. SEQ ID NO:26 contains a nucleic acid sequence spanning nucleotides 124 through 1953, referred to herein as nglmS-149_{1830}, which represents an open reading frame encoding a mutant glucosamine-6-phosphate synthase of the present invention, with an initiation codon spanning from nucleotides 124-126 and a termination codon spanning from nucleotides 1951-1953 of SEQ ID NO:26. The nucleic acid sequence of nglmS-149_{1830} is represented herein as SEQ ID NO:27. SEQ ID NO:27 encodes a mutant glucosamine-6-phosphate synthase protein of 609 amino acids referred to herein as GlcN6P-S-149, the deduced amino acid sequence of which is represented herein as SEQ ID NO:28.

The nucleic acid sequence of the 2184 base pairs between positions 1130 and 3313 of plasmid pKLN23-151 (these positions being determined relative to the equivalent positions in SEQ ID NO:13) are referred to herein as nucleic acid molecule nglmS-151_{2184}, the nucleic acid sequence of which is represented by SEQ ID NO:29. SEQ ID NO:29 contains a nucleic acid sequence spanning nucleotides 124 to 1953, referred to herein as nglmS-151_{1830}, which represents an open reading frame encoding a mutant glucosamine-6-phosphate synthase of the present invention, with an initiation codon spanning from

nucleotides 124-126 and a termination codon spanning from nucleotides 1951-1953 of SEQ ID NO:29. The nucleic acid sequence of nglmS-151_{1830} is represented herein as SEQ ID NO:30. SEQ ID NO:30 encodes a mutant glucosamine-6-phosphate synthase protein of 609 amino acids referred to herein as GlcN6P-S-151, the deduced amino acid sequence of which is represented herein as SEQ ID NO:31.

Strains isogenic to strain 2123-12 except for mutations conferring the gly→ser472 alteration were constructed using the scheme indicated in Fig. 7. Strains 2123-149 and 2123-151 were generated from plasmids pKLN23-149 and pKLN23-151, respectively. The presence of the mutation at position 2666 (SEQ ID NO:13) and the absence of mutations at positions 1367 and 2000 were verified by sequencing of PCR products from genomic DNA of these strains using the methods described in Example 8.

Example 10

This example compares properties of GlcN6P synthase enzymes from strains 2123-12, 2123-49, 2123-54, 2123-124, 2123-149, and 2123-151.

Strains 2123-12, 2123-49, 2123-54, 2123-124, 2123-149, and 2123-151, described in the examples above, were grown overnight in LB broth at 37°C then transferred to fresh LB broth. Cultures were grown to an absorbance at 600 nm of 0.8 to 0.9, then induced for GlcN6P synthase production by the addition of 1 mM IPTG. The cultures were grown for an additional three hours at 37° and harvested. Extracts were prepared from cells harvested from those cultures as described in Example 2 and were assayed for glucosamine-6-phosphate synthase using the spectrophotometric assay as described in

Example 2 except that a fructose-6-phosphate concentration of 20 mM was used. The enzyme was assayed in the presence and absence of added glucosamine-6-phosphate. In the absence of glucosamine-6-phosphate, the specific activities measured for these enzymes were similar except for that from strain 2123-124. The data from Table 6 suggests that the latter strain encodes a less active variant of the enzyme.

Table 6

Specific Activities of GlcN6P Synthase from Glucosamine-Producing Strains

Strain	Specific Activity, μmol min^{-1} mg^{-1}
2123-12	0.385
2123-49	0.375
2123-54	0.416
2123-124	0.0076
2123-149	0.494
2123-151	0.515

Fig. 10 shows that the GlcN6P synthase enzymes from strains 2123-49, 2123-54, and 2123-124 are significantly less inhibited by GlcN6P than the enzyme from strain 2123-12. Enzymes from strains 2123-149 and 2123-151 are slightly less inhibited by GlcN6P than the enzyme from 2123-12.

Thermal stability of the enzymes was also examined using these extracts. The extracts were incubated at 45°C (Fig. 11A) or 50°C (Fig. 11B) for various periods then assayed using the spectrophotometric assay. Figs. 11A and 11B show that the enzymes from 2123-49 and 2123-54 are much less stable than the wild type enzyme from strain 2123-12. The enzyme from strain 2123-124 is comparable in stability to the wild type enzyme,

and the enzymes from 2123-149 and 2123-151 are slightly less stable under the incubation conditions described here.

Example 11

The following example illustrates the effects of isopropylthio-β-D-galactoside (IPTG) concentration and temperature on glucosamine production.

Cultures of strains 2123-54 and 2123-124 were grown for 20 hours at 37°C on M9A medium (14 g/L K_2HPO_4, 16 g/L KH_2PO_4, 1 g/L $Na_3Citrate \cdot 2H_2O$, 5 g/L $(NH_4)_2SO_4$, pH 7.0) supplemented with 20 g/L glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and varying amounts of IPTG. At the end of the growth period, a sample was taken and the glucosamine concentration in the culture supernatant was assayed using the Elson-Morgan assay described in Example 2. The results shown in Fig. 12 indicate that the optimum IPTG concentration for production is about 0.2 mM.

Subsequently, strain 2123-54 was grown in the same medium as described above in shake flasks with either 0.2 or 1 mM IPTG and at 30°C or 37°C. These flask cultures were also fed glucose and ammonium sulfate as described in Example 4. At various intervals, samples were taken and the glucosamine concentrations in culture supernatants were assayed using the Elson-Morgan assay described in Example 2. Fig. 13 shows that under the conditions of this experiment, there was little difference in glucosamine production associated with the differences in IPTG concentration. However, growth at 30°C resulted in higher glucosamine production than did growth at 37°C. Results shown in Figs. 14A and 14B further indicated that at 30°C (Fig. 14A), glucosamine production continued after growth had ceased, while at 37°C (Fig. 14B), growth and glucosamine production occurred in concert.

When strains 2123-49 and 2123-124 were grown with 0.2 mM IPTG at 30°C, glucosamine production also occurred after growth had ceased, as shown in Figs. 15A (2123-49) and 15B (2123-124). As observed at 37°C, the highest concentrations of glucosamine were obtained with strain 2123-54, followed by 2123-124 and 2123-49. Also tested were strains 2123-149 and 2123-151 which produced negligibly higher concentrations of glucosamine than did 2123-12 (Table 7).

Table 7

Production of Glucosamine at 30°

Strain	Maximum Glucosamine Production, g/L
2123-12	0.3
2123-49	4.6
2123-54	7.2
2123-124	5.3
2123-149	0.6
2123-151	0.6

Example 12

The following example illustrates that glucosamine can be produced at higher concentrations in fermentor cultures of strain 2123-54 as compared to shake flasks. This example also illustrates that in fermentors, strain 2123-54 produces more glucosamine at 30°C than at 37°C.

Fermentation cultures of strain 2123-54 were cultivated in the medium shown in Table 8. Fermentations were run using NaOH for pH control to pH 6.7 and were fed a mixture of 33% glucose, 8% ammonium sulfate. Aeration and agitation were

adjusted to maintain a dissolved oxygen concentration of greater than 20% of air saturation.

Table 8

Fermentation Medium

Component	Amount, g/L
K_2HPO_4	14
KH_2PO_4	16
$Na_3Citrate\,2H_2O$	1
$(NH_4)_2SO_4$	5
$MgSO_4$	0.12
$CaCl_2$	0.011
Mazu 204 Antifoam	0.5 mL/L
IPTG	0.048
Glucose	20
Trace Metals	*

*Trace metal composition is 0.7 mg/L $CoCl_2$, 1.7 mg/L H_3BO_3, 0.6 mg/L $CuCl_2 2H_2O$, 10.5 mg/L $FeCl_3.6H_2O$, 12 mg/L $MnCl_2.4H_2O$, 1.5 mg/L $Na_2Mo_4.2H_2O$, 1.5 mg/L $ZnCl_2$.

In the following experiment, three fermentations were run in one-liter vessels containing an initial volume of 600 mL. Variables tested were as follows.

Fermentor #1: The mixture of 33% glucose and 8% ammonium sulfate was fed at such a rate that no glucose accumulated in the fermentor. Growth was at 37°.

Fermentor #2: As with fermentation #1 except that growth was at 30°.

Fermentor #3: As with fermentation #2 except that the feed rate was increased to maintain a constant glucose concentration in the fermentor of 5 to 10 g/L.

Results from these fermentations are shown in Figs. 16A, 16B and 16C. Comparison of the results from fermentors 1 (Fig. 16A) and 2 (Fig. 16B) shows that glucosamine titers are markedly higher at 30°C than they are at 37°C, as observed in shake flasks. The maximum glucosamine concentration observed was in the glucose-excess fermentor 3 grown at 30°C (Fig. 16C), at 10.9 g/L. At 30°C, growth and glucosamine concentration appeared to coincide, and there appeared to be a slight advantage to growth under glucose-excess. In subsequent fermentation experiments, run under conditions similar to fermentor #3, glucosamine concentrations in excess of 12 g/L have been obtained (data not shown).

In summary, the present inventors have described herein the use of metabolic engineering to create the first glucosamine overproducing strains of *E. coli*. The concept, proven here, will be generally applicable to any microorganism having a pathway for the production of amino sugars, or to any recombinant microorganism into which a pathway for the production of amino sugars has been introduced. In addition to the present strategy for creating a glucosamine-producing strain (i.e., eliminating glucosamine degradation and uptake and increasing expression of the *glmS* gene), the present inventors have also established that reducing product inhibition of glucosamine-6-phosphate synthase by glucosamine-6-phosphate improves glucosamine production.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. However, it is to be expressly understood that such modifications and adaptations are within the spirit

and scope of the present invention, as set forth in the following claims.